EMBRYOLOGICAL STUDY OF REPRODUCTIVE BARRIERS IN INTERSPECIFIC CROSSES BETWEEN *Carica papaya* L. AND *C. cauliflora* Jacq.

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ABSTRACT

The nature of sexual incompatibility between C. papaya L. and C. cauliflora Jacq. was examined. Pollen tube fluorescent staining studies revealed no inhibition of pollen tube development. Serial sections of developing hybrid-crossed ovules revealed significant post-zygotic abnormalities, with reciprocal hybrid differences. On C. papaya females pollinated by C. cauliflora, embryos aborted at a microscopic, undifferentiated stage beginning about the 45th day, normal endosperm was lacking, and intact pollen tubes persisted a shorter time than in intraspecific ovules. On C. cauliflora females pollinated by C. papaya, abortion was evident in some ovules by the 45th day, but in others polyembryony was observed, with differentiation ranging from none to fully differentiated. A minority of mature seeds yielded large, fully-formed multiple embryos; there appeared to be potential for in vitro germination. No endosperm was found. All embryos in both reciprocal hybrid crosses appeared to derive from the hybrid zygote, based on their orientation and location in the ovule.

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I. LITERATURE REVIEW

INTRODUCTION

Twenty-one species of Carica, all native to Central and South America, have been identified. In all of the species that have been examined, including C. papaya L. and C. cauliflora Jacq., the diploid chromosome number is eighteen (Badillo, 1968). The most important species from an economic standpoint is the papaya (Carica papaya L.), a fruit grown throughout the warm, tropical lowlands and frost-free, subtropical regions of the world. The melon-like fruits are borne on unbranched, herbaceous stems of short-lived, perennial trees crowned with large, palmately-lobed leaves. The species is polygamous, consisting of a stable female form and sexually unstable male and hermaphrodite types (Higgins and Holt, 1914; Storey, 1941, 1976).

While there is much genetic variation within the species C. papaya, there are also many potentially useful or interesting characteristics which are found only in other members of the genus. Several species have interesting fruit characteristics, including the appealing wine-red color of the exocarp in some types of C. goudotiana (Tr. et Planch) Solms and the pleasant aromas of C. stipulata Badillo, C. pubescens Lenne et Koch and C. cauliflora Jacq. Climatic adaptation of the species varies; C. parviflora (A. DC.) Solms appears to be more drought tolerant than C. papaya, while montane species such as C. stipulata and C. pubescens are adapted to cool conditions. Carica pubescens can tolerate light frosts which would be fatal to C. papaya. Carica monoica Desf. is monoecious, a quality which could be of value

in papaya by eliminating problems associated with sex segregation in dioecious and gynodioecious hermaphrodite types. Carica parviflora has a unique red flower color which could enhance the ornamental value of papaya.

From a papaya improvement standpoint, perhaps the most important quality to be found in the wild species is resistance to a serious disease, papaya ringspot virus (PRSV). This disease causes a rapid decline of the canopy, loss of production and eventual death of the plant. The epidemiology and symptomotology of PRSV has been described by Conover (1964). The disease is transmitted by aphids and spreads rapidly when vector population levels are high. No resistant papaya stocks have been found (Conover, 1964; Cook and Zettler, 1970), although Conover (1976) identified plants with some degree of tolerance, which was improved by selective breeding (Conover and Litz, 1978). Among the wild Carica species, resistance verified by inoculation studies has been reported for C. candicans A. Gray, C. cauliflora, C. heilbornii Badillo, C. pubescens, C. quercifolia (St. Hil.) Hieron. and C. stipulata (Malaguti et al, 1957; Ricelli, 1958; Conover, 1964a; Horovitz and Jimenez, 1967; Adsuar, 1971). Two of the species listed, C. cauliflora and C. quercifolia, were found susceptible by one author, but resistant by another. Horovitz and Jimenez (1967) attribute this to the fact that these species are very widely distributed and may each include susceptible and resistant genotypes.

Horovitz and Jimenez (1967) studied the inheritance of resistance to PRSV in crosses between susceptible and resistant species. The F1

hybrids were all resistant, but a small F2 population, and backcrosses to the susceptible parents, segregated in ratios suggesting that resistance is conferred by a single, autosomal, dominant gene.

The wild Caricas may also provide useful sources of resistance to other papaya diseases, although this aspect has not received as much attention as PRSV resistance. Patil and Wani (1980) reported C. cauliflora to be resistant to the leaf blight caused by Helminthosporium rostratum Dreschler while all of the papaya lines tested showed some degree of infection. The mode of inheritance was not examined.

In order to capitalize on the many useful characteristics in the genus Carica, breeders have been trying to produce interspecific hybrids between papaya and wild species by conventional means since the early part of this century. Higgins and Holt (1914) reported success crossing C. cauliflora X C. papaya and C. cundimarcensis (C. pubescens) X C. papaya, but no progeny of these crosses remain today. Reynolds (1959) initiated an apparently unsuccessful hybridization effort in Honduras based on reports that wild C. pennata (C. cauliflora) crossed spontaneously with domesticated papayas. Others who attempted to make these hybrids failed to obtain viable seeds (Larter, 1937,1938; Sawant, 1958; Mekako and Nakasone, 1975). Several reports have concluded that C. papaya is reproductively isolated from the rest of the Caricas (Jimenez and Horovitz, 1958; Horovitz and Jimenez, 1967; Mekako and Nakasone, 1975).

Attempts have been made to utilize in vitro embryo culture to overcome the reproductive barriers separating C. papaya from other

species in the genus. In Venezuela, F1 hybrids of C. papaya X C. pubescens, C. papaya X C. cauliflora, and C. papaya X C. stipulata were produced using embryo rescue techniques. The first-mentioned cross produced three vigorous, but sterile, female trees which were resistant to PRSV. The other two crosses were apparently lacking in vigor and did not survive transplanting into the field (Horovitz and Jimenez, 1967). The authors noted the absence of endosperm in hybrid ovules, and that hybrid embryos aborted before seeds matured, necessitating in vitro embryo rescue from immature ovules prior to embryo abortion. No firm evidence regarding genetic constitution of the offspring was given, but the fact that the plants were too weak to survive field planting is consistent with interspecific hybrid origin.

In Florida, Litz and Conover (1981) cultured immature ovules of C. papaya X C. cauliflora. Multiple embryos and embryogenic callus were produced. Polyembryony was also seen in some ovules in vivo 80-120 days after pollination. The authors believed that the embryos were somatic (asexual), originating from the inner integuments of the ovules, following abortion of the hybrid zygote due to failure of endosperm development. Plants were subsequently grown in pots in a greenhouse from the somatic embryos (Litz and Conover, 1982). In a subsequent report, Moore and Litz (1984) present isoenzyme marker evidence suggesting that the somatic embryos may actually have arisen from zygotic hybrid embryos.

Khuspe et al. (1980) of India reported successfully culturing F1 hybrid ovules of C. papaya X C. cauliflora. Five percent of the cultured ovules produced plants. The 24 plants which resulted were

PRSV resistant when inoculated. An F2 population of 3000 plants was produced, which segregated in a ratio of three resistant to one susceptible. The authors did not report any embryological details of ovule development, only that intact immature ovules produced plants *in vitro* that appeared morphologically to be hybrids. The plants were reportedly fertile and vigorous in the field, in contrast to those produced by Horovitz and Jimenez (1958). Subramanyam and Iyer (1982) reported normal meiosis in F1 *C. cauliflora* X *C. papaya*, lending support to the claim of fertile hybrids.

RESEARCH OBJECTIVE

The differing results and unclear interpretations of the above reports indicate a need for further study of developmental details of the seeds produced by interspecific pollination, in order to establish the nature of the interspecific incompatibility and the origin (somatic vs. zygotic) of embryos produced from interspecific crosses. The purpose of this thesis investigation is to attempt to identify the reason(s) for the difficulties that have been encountered when making crosses between *C. papaya* and *C. cauliflora*, and thereby clarify the interpretation of the earlier reports. If this can be accomplished, then ways to overcome the fertility barrier can be examined. There are several points to consider, including pollen germination and tube development, double fertilization and subsequent development of embryo and endosperm, and viability of hybrid offspring. The remainder of this literature review will attempt to summarize the various types of

barriers to interspecific hybridization that have been reported and the means that have been devised to overcome them.

EMBRYOLOGICAL DEVELOPMENT OF *Carica papaya* AND *C. cauliflora*

Detailed accounts of the fertilization process in angiosperms have been published by Maheshwari (1950), Jensen (1973) and Kapil and Bhatnagar (1975). The process is actually a double fertilization which begins with germination of the pollen grain on the stigma. A pollen tube grows into the stigma and through the style to the ovary where it enters an ovule through the micropyle. The tube penetrates the egg sac (or megagametophyte) through the filiform apparatus of one of the synergids. It then discharges its contents which contain the two sperm cells. One sperm nucleus enters the egg cell and fuses with the egg nucleus to form the zygote. The other sperm nucleus fuses with the two polar nuclei to form the 3n endosperm nucleus. The two fertilization events are called syngamy and triple fusion, respectively.

The endosperm serves to nourish the developing embryo. In some genera the endosperm is absorbed by the time the seed is mature, but in others, including *Carica*, the endosperm constitutes an important storage tissue in the mature seed. In the latter case, the nutrients are absorbed by the embryo during the germination process.

Traub and O'Rork (1939) examined pollen tube growth in *C. papaya*. They observed that "the pollen tubes grow from the stigma through the central region of the style to the apical end of the ovarian cavity, and then emerge into, and grow on, the surface of the ovarian cavity to the ovules". The time required for the pollen tubes to reach the

ovules ranged from about one and one-half days for the apical end to about five days for the proximal end. Foster (1943) made a detailed study of reproduction in papaya. She confirmed earlier reports (Usteri, 1907; Kratzer, 1918) that the megagametophyte is of the seven-celled, eight-nucleate type. At about 10 days after pollination, the pollen tubes entered the ovules, but fertilization did not occur until 13-15 days had elapsed. The zygote persisted for 5-8 days while free-nuclear endosperm began development. At about 23-30 days after pollination, a four-celled embryo was formed, accompanied by free-nuclear endosperm and elongated nucellar cells in the chalazal end of the ovule. Foster speculated that the peculiar nucellar cells could have a nutritional function for the developing endosperm. At 32-35 days the embryo was eight-celled; at 64 days it was many celled with a suspensor-like projection on one side. Foster believed the suspensor could have a haustorial function. The pollen tube was still prominent and closely pressed against the embryo at this stage, while the endosperm was just beginning to make contact with the embryo. At 79 days, the parts of the embryo had differentiated and the endosperm was making the transition from free-nuclear to cellular. At 4 months the embryo was nearly mature and the endosperm was cellular.

Foster interpreted her findings as follows: "The persistence of the pollen tube, the lag in development of the embryo, and the separation of the young embryo from the endosperm, together with the presence of a suspensor might suggest an unusual nutritive relationship. Although more work is needed to determine the exact interrelationships, it might be suggested that the persistent pollen

tube may be closely associated with the nutrition of the embryo in its early stages of development. As the embryo grows older and is more embedded in the endosperm, there then may occur a shift in the nutritive arrangement and the endosperm might become more functional in relation to the embryo."

Lamoureux (1955) examined seed development in papaya in connection with a study of an apparent embryo-lethal factor observed in embryos with a pair of dominant alleles for sex determination. The male and hermaphrodite alleles are dominant to the female allele; all males and hermaphrodites are therefore heterozygous for sex-determining alleles, due to the lethality of the homozygous dominant genotypes. Lamoureux crossed hermaphrodites with males, a cross which should produce the lethal combination in approximately 25% of the embryos produced. The remaining 75% of the embryos should all be normal, consisting of equal proportions of males, hermaphrodites and females. (Foster's (1955) study used a female X male cross which should not produce the lethal genotype.)

Lamoureux observed normal development in approximately 75% of the embryos examined, as predicted. The development paralleled Foster's description for the most part, except that the events of the first month after pollination were accelerated by several days. This may have been due to a difference in temperature, since Foster grew her plants in a Wisconsin greenhouse, which may have been considerably cooler than the Hawaiian climate where Lamoureux worked.

Lamoureux could distinguish two types of embryos beginning about 60 days after pollination. The first type was the normal one; the

second type was apparently the result of the embryo lethal factor. The abnormal development was characterized by a lack of suspensor elongation and failure of the endosperm to make contact with the embryo, while the nucellus was more persistent and in close contact with the embryo. The pollen tubes were still in close contact with the embryos at 66 days, but had disintegrated in both types of ovules by 80 days. By 100 days, all of the abnormal embryos were dead or nearly so, the endosperm and nucellus were disintegrating and some of the ovule walls had started to collapse. At 120 days, the abnormal ovules were entirely empty, although in many cases the integuments continued to develop to maturity.

Based on the observation that the time of pollen tube disintegration coincides with the commencement of abortion of the embryo and endosperm in the abnormal ovules, Lamoureux concluded that the lethal factor acts by preventing a necessary shift in the nutritive arrangement for the embryo from dependence on nutrients transported via the persistent pollen tube, and possibly the nucellus, to dependence on the endosperm as the embryo becomes larger and differentiates.

If the "nutritive shift" hypothesis is correct, it may help explain the failure of Carica interspecific hybrid embryos to mature, since the endosperm fails to develop in these crosses (Jimenez and Horovitz, 1958). Consequently, no shift to nutrition from the endosperm is possible, and the embryo would perish when the pollen tube disintegrates. In Venezuela, interspecific hybrid embryos in papaya ovules pollinated with several species, including C. cauliflora, attained sufficient size to be cultured in vitro by 3 months after

pollination, but were aborting at about 3 1/2 months (Jimenez and Horovitz 1958). This time frame corresponds fairly well with the time for the proposed shift from nutrition via the pollen tube to nutrition from the endosperm. Further support for this hypothesis comes from an intraspecific cross of a diploid with a tetraploid papaya (de Zerpa 1958). As with the interspecific hybrids, no endosperm formed, and the young triploid embryos could only be grown by in vitro culture.

Reproduction in C. cauliflora, the other species to be used in this study, has not received the attention given to papaya. Jimenez and Horovitz (1958) discussed it briefly in reference to interspecific hybridization with papaya. The time required for fruit maturation after pollination of a flower is about 6 months in both species. When C. cauliflora was used as the female parent in crosses with papaya, the embryos were more precocious than in the reciprocal, reaching sufficient size to be cultured in vitro at 2 1/2 months and aborting at 3 months.

BARRIERS TO REPRODUCTION IN INTERSPECIFIC CROSSES

Pre-zygotic barriers: Pre-zygotic barriers to interspecific hybridization in angiosperms include any factors which prevent the completion of the normal sequence of events from the time of pollination until double fertilization is accomplished. As mentioned in the previous section, fertilization is the result of a complex series of events. Consequently, there are many points at which reproductive barriers can occur. The types of abnormalities which have been observed in wide crosses, as well as the possible causes behind

them, will be reviewed in this section. The first phase in the fertilization process is the hydration and germination of the pollen grains on the stigma. For this to occur, the stigma must provide the necessary stimuli to the pollen to trigger its development. These stimuli are apparently specific within related groups of plants; therefore, the recognition response often fails to function properly in wide crosses, as the following example will illustrate: Knox et al. (1976) examined pollen behavior in two wide crosses on Gladiolus. The first cross was an intrafamilial one with Crocosmia. The Crocosmia pollen grains hydrated and germinated on the Gladiolus stigma, but the pollen tubes were unable to enter the papillae on the stigmatic surface. The second cross was an interfamilial one with Gloriosa. The Gloriosa pollen grains failed to hydrate or respond to the Gladiolus stigma. Shivanna (1982) attributed the pollen behavior in these crosses to passive rejection due to the lack of some substance(s) on the stigma needed for normal function of the pollen. Apparently the intrafamilial cross on Gladiolus had the necessary substance(s) for hydration and germination, but not for penetration, of the pollen tubes into the stigma, while the more distant interfamilial cross lacked even the first substance required.

The next phase of fertilization is the growth of the pollen tube through the style. This growth can be arrested in both intra- and interspecific pollinations. Several different systems of intraspecific incompatibility are known; for comprehensive reviews see Heslop-Harrison (1975) and Shivanna (1982). The important point for this discussion is that intraspecific incompatibility is an active,

genetically-controlled response mechanism by which the pistil rejects certain genotypes of pollen in order to prevent inbreeding. The rejection can be manifested at any point between pollen hydration and fusion of the gametes, depending on the type of self-incompatibility (SI) genes involved.

Interspecific incompatibility in closely-related species can sometimes be attributed to identical SI genes derived from a common ancestral species (Lewis and Crowe, 1958). This is especially likely to be true if the incompatibility is unilateral between self-compatible and self-incompatible species.

In other cases, interspecific incompatibility can be attributed to fundamental differences in the genomes of the two species as a result of divergent evolution (Heslop-Harrison, 1975; Hogenboom, 1975). These differences result in physiological incompatibility between the microgametophyte and the stylar tissue which is manifested by an inhibition of pollen tube growth. Hogenboom (1975) characterized this passive rejection phenomenon as an "incompleteness of relationship" for which he proposed the term "incongruity", reserving the term incompatibility for active rejection due to SI genes. Heslop-Harrison (1975) notes that the presence of a SI system in a cross may have no bearing on the true isolation mechanism between the two species; there may also be incongruity which is masked by the SI system. A very simple, but graphic, example of incongruity is seen in crosses between a female of a species with very long styles and pollen from a species with very short styles. The pollen may not be adapted to grow the additional length required, even if the gametes have the potential to

form viable offspring if fertilization were to take place (Allard, 1966).

The final category of pre-zygotic barrier is failure of double fertilization. Bannikova and Khedynich (1974) reviewed examples of abnormalities in double fertilization of interspecific hybrid crosses including differences in the rate and synchronicity of processes, failure of syngamy and/or triple fusion, and the appearance of structural irregularities in sperms. While it is probable that these phenomena are most often due to incongruity, there are also some genera, e.g. Theobroma, in which SI genes prevent nuclear fusion after the sperms have entered the egg sac (Bennet and Cope, 1959); consequently, incompatibility may also play a role in certain cases.

Post-zygotic barriers: Post-zygotic barriers to interspecific hybridization can generally be attributed to genetic disharmonies of various types. This topic has been thoroughly reviewed by Stebbins (1958).

1.) Disharmony between genomes of the parental species. The embryo may develop abnormally or abort due to general incompatibility between the parental genomes (Stebbins, 1958). The resulting degeneration tends to be expressed as a failure to successfully undergo some critical period of differentiation of tissues. For example, McCray (1933) identified three distinct stages at which embryo breakdown was likely in hybrid embryos of Nicotiana species: a.) the four-to-eight cell pro-embryo, b.) the differentiation of the vegetative growing point, and c.) germination.

Some instances of embryo breakdown are due to the action of single genes whose lethal alleles are only expressed in interspecific hybrids (Hollingshead, 1930; Sears, 1944). However, Stebbins (1958) points out that these simple Mendelian factors are not true barriers to interspecific hybridization, since some of the genotypes do not inherit the lethal allele. The species involved are generally well-isolated from each other by other factors as well.

Stebbins (1958) reviewed examples of tumor formation and abnormal F1 phenotypes related to specific hybrid genotypes and ploidy levels, as well as the effect of trisomy on crossability.

2.) Disharmony between the genome of one species and the cytoplasm of another. There are many reports of unilateral incompatibility attributable to cytoplasmic differences. The cytoplasmic incompatibility can result in non-viability, weakness or sterility of the F1. Stebbins (1958) reviews the earlier work on this topic, with additional examples provided by Stalker (1980). (If cytoplasmic incompatibility were to exist between the hybrid nucleus and both parental cytoplasms, it would be indistinguishable from general genomic incompatibility.)

3.) Disharmony between the zygote and surrounding tissues. Embryo non-viability in interspecific hybrids can be due to lack of development of the endosperm or maternal tissues; this was clearly demonstrated by Laibach (1925), who discovered that Linum species hybrids could be produced by in vitro culture of embryos excised before seed collapse and abortion occurred.

The importance of genetic harmony between embryo, endosperm and maternal tissue was demonstrated in cotton (Weaver, 1957). It was observed that ovules of interspecific hybrids would develop normal endosperm and maternal tissues only if the embryo failed to develop. Consequently, any developing embryos would abort due to lack of supporting tissues.

Ploidy level can influence the viability of an interspecific cross independently of other factors. For example, Cooper and Brink (1945) found that a diploid cross of Lycopersicon pimpinellifolium (2n) X L. peruvianum (2n) failed, yet the same two species produced viable seeds when crossed L. pimpinellifolium (4n) X L. peruvianum (2n), even though intraspecific L. pimpinellifolium 4n X 2n crosses failed.

A hypothesis of endosperm balance number (EBN) has been proposed by Johnston et al (1980) to explain endosperm development in interspecific crosses. It was observed from interploidy-intraspecific crosses that normal endosperm development was dependent on a balanced 2:1 ratio of maternal:paternal chromosomes in the endosperm. Although this condition cannot be applied directly to interspecific crosses, a system was devised whereby the genome of each species was assigned a specific value (EBN) in the endosperm. Once this EBN was determined for each member of a genus, the fertility of any cross could be predicted on the basis of the EBN conforming to the 2:1 ratio in the endosperm. In the Lycopersicon example in the preceding paragraph, L. pimpinellifolium (2n) would have an EBN of 1, while L. peruvianum (2n) would have an EBN of 2. Consequently, only L. pimpinellifolium (4n) (EBN=2) was fertile crossed with L. peruvianum (2n).

Another category of maternal:hybrid incompatibility is termed somatoplastic sterility. Cooper and Brink (1940) observed that some Nicotiana species hybrid seeds abort due to impaired growth of the endosperm caused by excessive thickening of the nucellus or inner integument. The hyperplasia isolates the endosperm and cuts off its supply of nutrients, which in turn starves the embryo. This phenomenon was first observed and named by Brink and Cooper (1939) in self-incompatible matings of Medicago sativa.

A phenomenon related to somatoplastic sterility was observed by Rappaport et al (1950) in Datura interspecific crosses. In this case, ovular tumors develop from the endothelium. These tumors produce an auxin-like substance which inhibits embryo development (Rietsema et al., 1954).

4.) Hybrid sterility or genetic breakdown in subsequent generations. After producing a viable F1 interspecific hybrid, plant breeders often encounter further barriers to gene transfer between the species due to sterility of the F1 or genetic breakdown in subsequent generations. Stebbins (1958) reviewed these phenomena in detail, and Stalker (1980) reviewed various examples of chromosome elimination in subsequent generations due to genomic disharmony, but these aspects will not be considered further for this thesis because the scope is limited to barriers to the formation of F1 hybrids.

II. MATERIALS AND METHODS

PARENTAL GENOTYPES

To limit the effects of genetic variation in the study, only one strain of each species was used. The C. papaya parent was a selection known as 'Washington' (UH accession # 417) from Bangalore, India. It is dioecious, bearing yellow flowers and yellow-fleshed fruits weighing approximately 1-2 kilograms. The stems, petioles and peduncles display an intense purple pigmentation. Height at 2 years of age is approximately 4-5 meters.

The C. cauliflora type used (UH accession # 345) originated in Venezuela. It is dioecious, bearing white flowers and orange-skinned, white-fleshed fruits weighing 100-200 grams. The fruit is considered inedible but has a pleasant fruity aroma. Flowers form repeatedly at the older nodes down to the base of the trunk. The non-branching trees reach approximately 2 meters in height at 2 years of age. Unlike C. papaya, the trees, especially the females, drop most of their leaves and become semi-dormant from January to March in Hawaii.

GROWING CONDITIONS

The plants were grown at Poamoho Agricultural Research Station on the island of Oahu. The station is 200 meters above sea level. Mean temperature for 1985 varied from 20 C in January to 24 C in July. The soil is an oxisol (Wahiawa series). Average annual rainfall is 89 millimeters. Furrow irrigation was provided, occasionally limited by

water shortages. The C. papaya seedlings were set out in the field in November of 1983 and C. cauliflora in May of 1984.

POLLEN TUBE STUDIES

To compare pollen tube behavior of the parent species in intraspecific and interspecific situations, controlled interspecific crosses were made reciprocally, as well as intraspecific pollinations of both species. In addition, flowers on female trees of both species were bagged before anthesis for use as unpollinated controls. Because the trees used were dioecious, no emasculation was necessary.

Care was taken to avoid uncontrolled pollination. Crosses were made either on mature female buds just prior to opening or on freshly-opened female flowers which had been bagged before they opened. Pollen was obtained from mature, unopened male flowers with dehisced anthers. Liberal amounts of pollen were applied to the stigmas by brushing the anthers on the stigma. To avoid cross-contamination, hands were rinsed with 95% ethanol when switching from one species of pollen to the other. Flowers were bagged and labelled immediately after pollinating. Unpollinated control flowers were bagged and labelled prior to opening.

The pollinations were made in May and June of 1986. The flowers were harvested 7 days after pollination. The types and numbers of crosses made are listed in Table 1.

Carica pollen tubes are transparent and require staining for good visibility. Currier (1957) first reported the value of water-soluble aniline blue staining for viewing pollen tubes. The tubes are rich in

TABLE 1. -- Numbers of intraspecific pollinations, interspecific pollinations and unpollinated controls made for pollen tube growth study

Cross	# Made
C. papaya X *C. papaya*	4
C. cauliflora X *C. cauliflora*	3
C. papaya X *C. cauliflora*	7
C. cauliflora X *C. papaya*	4
C. cauliflora unpollinated	3
C. papaya unpollinated	3

callose which, when stained, fluoresces a bright yellow-green color under ultraviolet light in a darkened room; in contrast, the surrounding tissue is low in callose and appears dull violet or gray.

For this investigation a modified staining technique was devised for viewing Carica pollen tubes in fresh material. The position of the tubes on the inner wall of the ovary cavity (Traub and O'Rork 1939) makes it possible to view them in situ. The C. papaya pistils were bisected longitudinally to expose the pollen tubes. The ovaries of C. cauliflora are composed of five false locules instead of a single open cavity as in C. papaya; consequently after bisecting the pistils, the locules were carefully pried open as necessary to expose the pollen tubes located on the inner surface of individual locules.

To darken the background for maximum contrast in photomicrographs, the specimens were first treated for 5-10 minutes with iodine-potassium iodide solution. This solution was made from 1 gram of iodine crystals and 1 gram of potassium iodide crystals in 100 milliliters of 80% ethanol. After draining briefly, the specimens were stained for 10-20 minutes with a 0.1% solution of water-soluble aniline blue dye in 0.1 N tribasic potassium phosphate (Martin 1959). For staining, each specimen was positioned with the cut side facing up and the ovarian cavity was filled with stain, using a Pasteur pipette, until the cut surface was covered with stain.

Immediately after staining, the specimens were drained and then viewed under long wave (366nm) ultraviolet light in a darkened room. A Zeiss DRC stereomicroscope fitted with an MC 63 photomicrographic 35 mm camera was used for viewing and recording the findings.

Photomicrographs were made with Kodak Tri-X Pan film (ASA 400) exposed at Exposure Index 1600. The film was pushed in development using XR-1 developer manufactured by Perfection Photographic Products, Inc., Los Angeles, California 90064.

FIXED AND STAINED OVULE SECTIONS

To compare ovule development resulting from intraspecific and interspecific pollinations, the parental species were pollinated in all combinations, exercising the same precautions against contaminating pollen as for the preceding pollen tube study. Fruits were harvested at 9 stages of development, ranging from 3 days through 90 days after pollination. Due to constraints on the availability of crossing materials and the remoteness of the fields from the laboratory (45 km), it was not possible to make all of the pollinations at the same time nor to have all of the harvest intervals correspond exactly. One or two fruits of each stage were harvested; the harvested fruits tended to be the best developed ones whenever there were several to choose from. Any remaining fruits were allowed to reach maturity, except those from C. papaya X C. cauliflora, which were mainly harvested after 3-4 months (see Table 3). The crosses for staining and sectioning, together with the date of pollination and age at harvest, are listed in Table 2.

For ease of handling, the younger material was prepared as sections of ovary with ovules attached; included were the pistils (intraspecific and interspecific crosses) of C. cauliflora at 3,9,14 and 22 days after pollination and of C. papaya at 3 and 9 days after

TABLE 2. -- Crosses made for serial sections, date of pollination, age at harvest and number of ovules sectioned

Cross	Date of pollination	Age (days)	# Ovules sectioned
C. papaya X *C. papaya*	05-24-85	3	*
C. papaya X *C. papaya*	03-12-85	9	*
C. papaya X *C. papaya*	05-07-85	16	8
C. papaya X *C. papaya*	04-23-85	23	6
C. papaya X *C. papaya*	04-23-85	30	8
C. papaya X *C. papaya*	05-07-85	44	2
C. papaya X *C. papaya*	12-30-84	60	2
C. papaya X *C. papaya*	12-13-84	75	2
C. papaya X *C. papaya*	12-13-84	90	16
C. cauliflora X *C. cauliflora*	05-24-85	3	*
C. cauliflora X *C. cauliflora*	04-30-85	9	*
C. cauliflora X *C. cauliflora*	04-23-85	16	*
C. cauliflora X *C. cauliflora*	04-16-85	23	*
C. cauliflora X *C. cauliflora*	04-16-85	30	8
C. cauliflora X *C. cauliflora*	03-08-85	45	4
C. cauliflora X *C. cauliflora*	04-12-85	62	4
C. cauliflora X *C. cauliflora*	03-08-85	75	2
C. cauliflora X *C. cauliflora*	03-08-85	90	2

* Sectioned piece of ovary with at least 10 ovules attached

TABLE 2. -- (Continued) Crosses made for serial sections, date of of pollination, age at harvest and number of ovules sectioned

Cross	Date of pollination	Age (days)	# Ovules sectioned
C. papaya X C. cauliflora	05-24-85	3	*
C. papaya X C. cauliflora	03-12-85	9	*
C. papaya X C. cauliflora	05-07-85	16	10
C. papaya X C. cauliflora	04-30-85	23	>25
C. papaya X C. cauliflora	04-16-85	30	9
C. papaya X C. cauliflora	04-16-85	45	25
C. papaya X C. cauliflora	12-06-84	60	9
C. papaya X C. cauliflora	11-29-84	75	9
C. papaya X C. cauliflora	02-07-85	90	9
C. cauliflora X C. papaya	12-17-85	3	*
C. cauliflora X C. papaya	04-30-85	9	*
C. cauliflora X C. papaya	06-13-85	14	*
C. cauliflora X C. papaya	06-21-85	22	*
C. cauliflora X C. papaya	06-13-85	28	12
C. cauliflora X C. papaya	06-21-85	44	12
C. cauliflora X C. papaya	06-21-85	62	12
C. cauliflora X C. papaya	07-11-85	76	12
C. cauliflora X C. papaya	04-18-85	90	9

* Sectioned piece of ovary with at least 10 ovules attached

pollination. All of the older ovules were removed from the fruits prior to fixing.

The material was fixed immediately after harvest according to a modification of Foster's (1943) technique. The samples were first placed in Craf III fixative solution with a drop of Kodak Photo-Flo 200 wetting agent added to aid penetration. The vials were placed in a vacuum aspirator for 10-15 minutes to remove gas bubbles. After a few hours the solution was changed using the same fixative without wetting agent.

The fixed specimens were dehydrated in a tertiary butyl alcohol series and embedded in paraffin. Serial sections were made on a rotary microtome at 11 micrometers thickness, then mounted on slides with Haupt's gelatin solution and dilute formaldehyde. The mounted sections were stained with Delafield's haematoxylin and made permanent with synthetic resin and glass cover slips.

Examination of slides and photomicrography was done on a Zeiss Standard microscope equipped with an MC 63 35 mm camera. Photomicrographs were made using Kodak Technical Pan Film 2415 at Exposure Index 25. A green filter was used in the microscope to improve contrast.

DISSECTION OF FRESH SEEDS

Fruits produced through reciprocal crosses of C. cauliflora and C. papaya were opened, beginning at 76 days after pollination to full maturity (6 months), and seeds, when present, were individually

dissected using a dissection microscope. Contents of fruits and individual seeds were recorded. The numbers of fruits examined, together with ages and harvest dates, are listed in Table 3.

TABLE 3. -- Number of fruits of interspecific crosses examined for seed dissection, with date of pollination and age at harvest

Cross	Date of pollination	Number harvested	Age at harvest (days)
C. cauliflora X *C. papaya*	10-10-85	1	153
C. cauliflora X *C. papaya*	10-10-85	1	165
C. cauliflora X *C. papaya*	10-10-85	1	172
C. papaya X *C. cauliflora*	10-11-84	9	116
C. papaya X *C. cauliflora*	10-18-84	6	109
C. papaya X *C. cauliflora*	10-23-84	1	90
C. papaya X *C. cauliflora*	10-23-84	1	104
C. papaya X *C. cauliflora*	10-25-84	5	102
C. papaya X *C. cauliflora*	11-01-84	6	96
C. papaya X *C. cauliflora*	11-29-84	2	76
C. papaya X *C. cauliflora*	12-06-84	4	99
C. papaya X *C. cauliflora*	12-13-84	1	92
C. papaya X *C. cauliflora*	03-07-85	3	180

III. RESULTS

POLLEN TUBE GROWTH IN INTRA- AND INTERSPECIFIC MATINGS

Results of the pollen tube fluorescence study on C. papaya pistils are shown in Figures 1-3. Each photograph shows the inner surface of a bisected and stained pistil 7 days after the date of pollination. The base of the style is situated at the top center and a few of the uppermost ovules are visible as large, indistinct lighter bodies at the bottom of each photograph.

Typical pollen tube development in intraspecific C. papaya is shown in Figure 1. The pollen tubes can be seen as fine white lines fanning out over the inner surface of the ovary wall from the style to the ovules. Callose plugs in the pollen tubes appear as brighter flecks. Most of the tubes extend straight toward the ovules, but on close inspection one can discern an occasional tube at right angles to the rest. By contrast, an unpollinated C. papaya pistil, used as a pollination control, is shown in Figure 2. No pollen tubes are evident, nor were any visible in three other unpollinated control pistils examined.

Typical pollen tube development in C. papaya pollinated by C. cauliflora is shown in Figure 3. Pollen tubes can be seen extending to the ovules at the bottom of the photograph. No evidence of pollen tube inhibition was observed.

Pollen tube development in C. cauliflora pistils is shown in Figures 4-6. Each photograph shows part of a bisected and stained

Figure 1

Carica papaya pistil with fluorescing pollen tubes, longitudinal section, 7 days after intraspecific pollination (X100)

Figure 2

Unpollinated Carica papaya pistil 7 days after anthesis, longitudinal section (X100)

Figure 3

Carica papaya pistil with fluorescing pollen tubes 7 days after pollination by C. cauliflora, longitudinal section (X100)

Figure 4

Carica cauliflora pistil with fluorescing pollen tubes 7 days after intraspecific pollination, longitudinal section (X65)

Figure 5

Unpollinated Carica cauliflora pistil 7 days after anthesis, longitudinal section (X65)

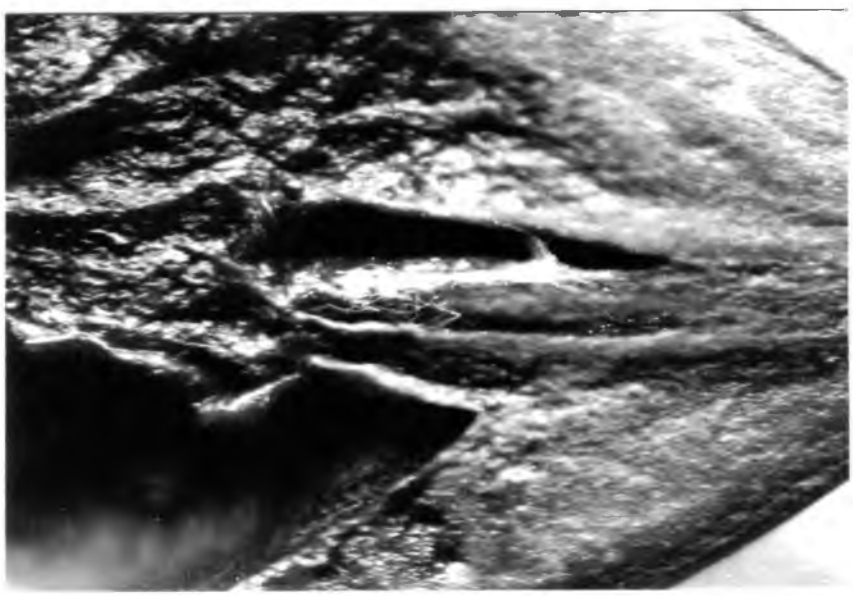

Figure 6

Upper portion of Carica cauliflora ovary with fluorescing pollen tubes 7 days after pollination by C. papaya, longitudinal section (X65)

pistil 7 days after the date of pollination. The orientation of the pistil is horizontal with the base of the style on the right.

Pollen tube development in intraspecific C. cauliflora is shown in Figure 4. Pollen tubes appear as twisted white threads near the ovules. The irregular inner surface of the C. cauliflora pistil with its five false locules reduces the visibility of the pollen tubes compared to the C. papaya specimens.

An unpollinated C. cauliflora pistil is shown in Figure 5. No pollen tubes are evident nor were any found in three other control pistils examined.

Development of C. papaya pollen tubes in a C. cauliflora pistil is shown in Figure 6. Pollen tubes are visible in the center of the photograph in the area where the false locules converge into a small cavity.

Regardless of the pollen source, pollinated pistils of both species were found to have pollen tubes extending to the lowest ovules in the pistils after 7 days. (The photographs were taken at the stylar ends of the pistils because the pollen tubes are most visible there.)

OVULE DEVELOPMENT IN INTRA- AND INTERSPECIFIC MATINGS

The photographs in Figures 7-84 record the development of ovules of the intraspecific parental lines and the interspecific hybrid crosses. Unless otherwise indicated, the sections are longitudinal with the micropylar end of the ovule on the right and the chalazal end on the left. Also, certain structures were considerably distorted in

the preparation of the specimens; the endosperm in particular tended to collapse and fragment.

Intraspecific C. papaya: Ovules at 3 days after pollination showed no evidence of fertilization or penetration by pollen tubes. The micropyles were open and the egg apparatus was intact, as indicated by the intact synergids (Figure 7). The synergids would be expected to degenerate rapidly after penetration by a pollen tube (Foster 1943).

On the 9th day after pollination, pollen tubes had reached the egg and syngamy had occurred. Figure 8 shows the pollen tube extending the length of the micropyle and in contact with the newly-formed zygote. The remains of a synergid are visible near the zygote. No endosperm was detected at this stage.

On the 16th day the zygote persisted in some ovules (Figure 9) but in others it had begun to divide, as evidenced by the two-cell pro-embryo (Figure 10). Also clearly visible in Figure 10 is the very twisted and bulbous pollen tube. At this stage there was already abundant free-nuclear endosperm (Figure 11).

On the 23rd day the embryo was still at the two-cell stage, but the free-nuclear endosperm had greatly increased along with the rest of the ovule. Figure 12 shows the endosperm layer inside of the thick nucellus; the pro-embryo is not included in this section but appeared essentially the same as the two-cell pro-embryo in the 16-day material.

By the 30th day the embryo had increased to approximately 4-8 cells. The pollen tube remained prominent and in close contact with the embryo (Figure 13). The rest of the ovule had increased somewhat

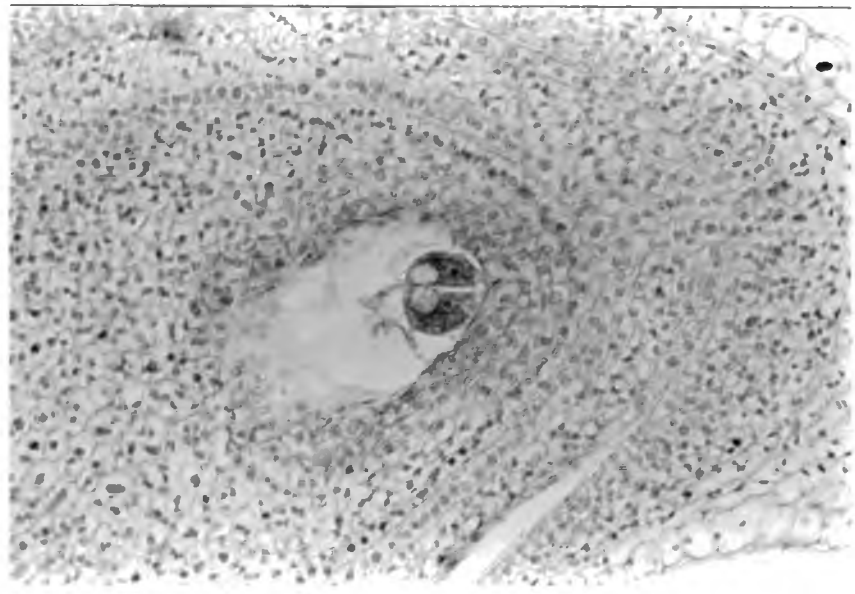

Figure 7

Carica papaya ovule 3 days after intraspecific pollination, with pair of intact synergids (X785)

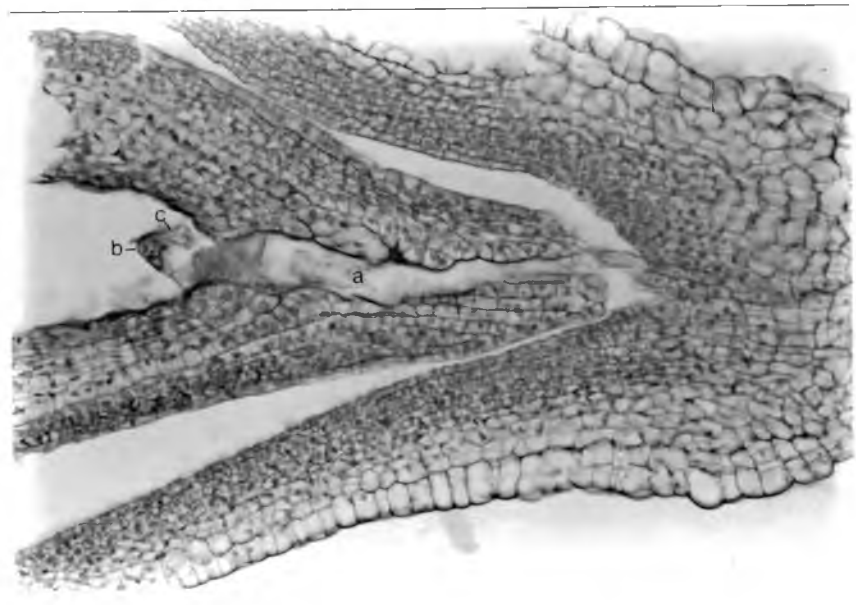

Figure 8

Carica papaya ovule 9 days after intraspecific pollination, with pollen tube (a), zygote (b) and remains of synergids (c) (X785)

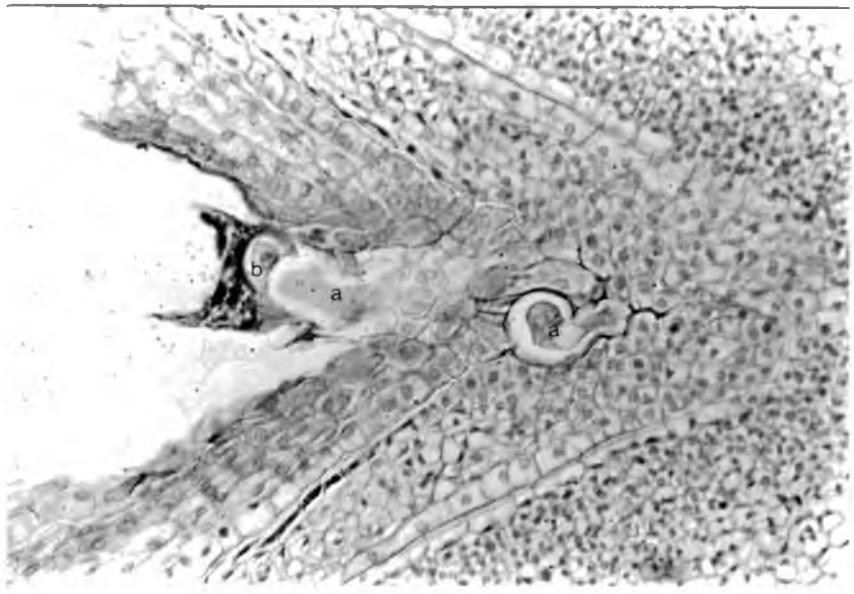

Figure 9

Carica papaya ovule 16 days after intraspecific pollination, with portions of pollen tube (a) and zygote (b) (X785)

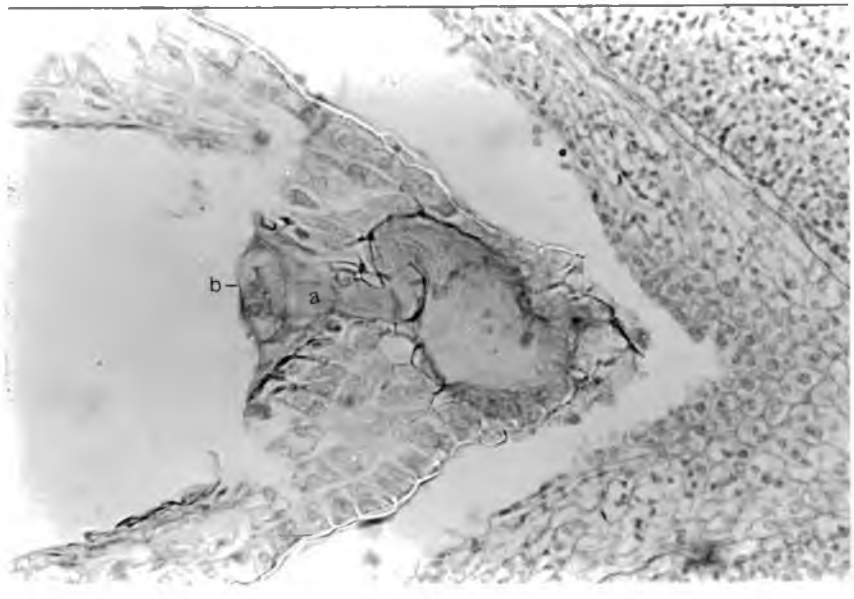

Figure 10

Carica papaya ovule 16 days after intraspecific pollination, with end of pollen tube (a) in contact with pro-embryo (b) (X785)

Figure 11

Carica papaya ovule 16 days after intraspecific pollination, with free-nuclear endosperm (a) (X315)

Figure 12

Carica papaya ovule 23 days after intraspecific pollination, whole ovule with free-nuclear endosperm (a) inside of nucellus (b) (X78)

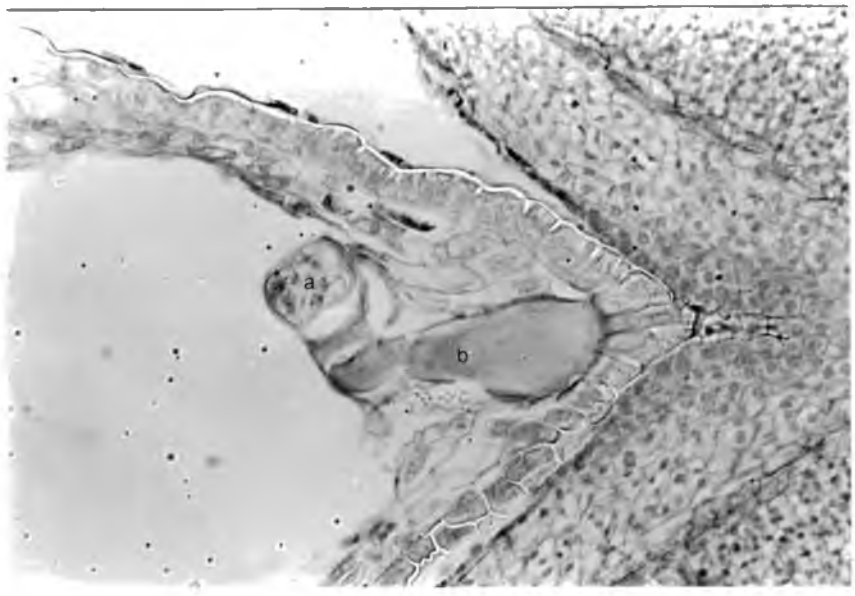

Figure 13

Carica papaya ovule 30 days after intraspecific pollination, with embryo (a) and end of pollen tube (b) (X785)

Figure 14

Carica papaya ovule 30 days after intraspecific pollination, whole ovule showing endosperm (a) (X78)

in overall size, with the endosperm layer thickening but still free nuclear and still surrounded by a thick layer of nucellus (Figure 14).

On the 44th day, the embryo was still very small, on the order of 16 or more cells (Figure 15), while the rest of the ovule had increased considerably in size (Figure 16). The endosperm had thickened substantially, and it stained more densely, but was still free nuclear. The nucellus was still present, but it represented less of the total mass of the ovule than in earlier stages; the appearance of the nucellus was spongy due to cell enlargement. On close inspection of Figure 16, the intact pollen tube can be seen on the right.

By the 60th day, the embryo had increased only slightly in size and remained undifferentiated (Figure 17). The pollen tube was intact and in contact with the embryo. The endosperm was still free nuclear but had thickened, especially at the chalazal and micropylar ends (Figure 18). In the nucellus, individual cells appeared to be breaking down.

On the 75th day, the embryo still consisted of only a few dozen cells but was acquiring polar differentiation (Figure 19). The tip of the pollen tube was still evident, forming a bulbous reservoir at the base of, and in contact with, the suspensor of the embryo. The endosperm had begun to turn cellular at the micropylar end, but was mostly free nuclear in the rest of the ovule; it still formed a relatively thin layer inside of the partially collapsed nucellus (Figure 20).

By the 90th day, the ovules showed a range of maturity. The least-developed ovule had a small, club-shaped embryo with a well-developed

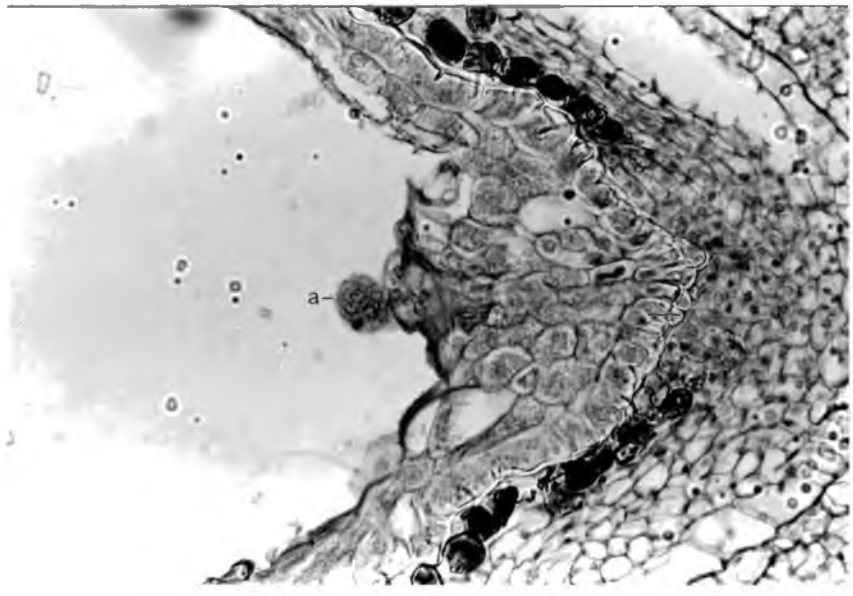

Figure 15

Carica papaya ovule 44 days after intraspecific pollination, with embryo (a) (X785)

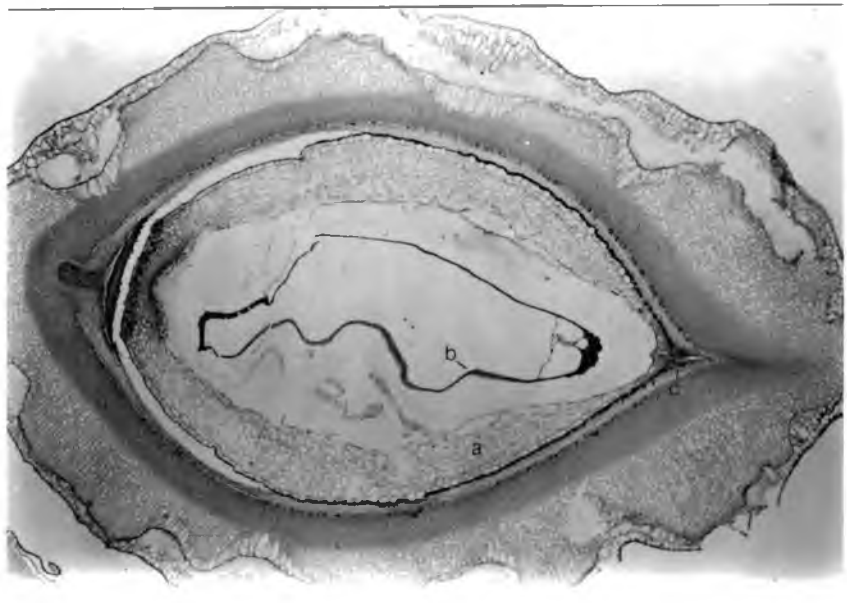

Figure 16

Same ovule as Figure 15, adjacent section with nucellus (a), endosperm (b) and barely-visible pollen tube (c) (X78)

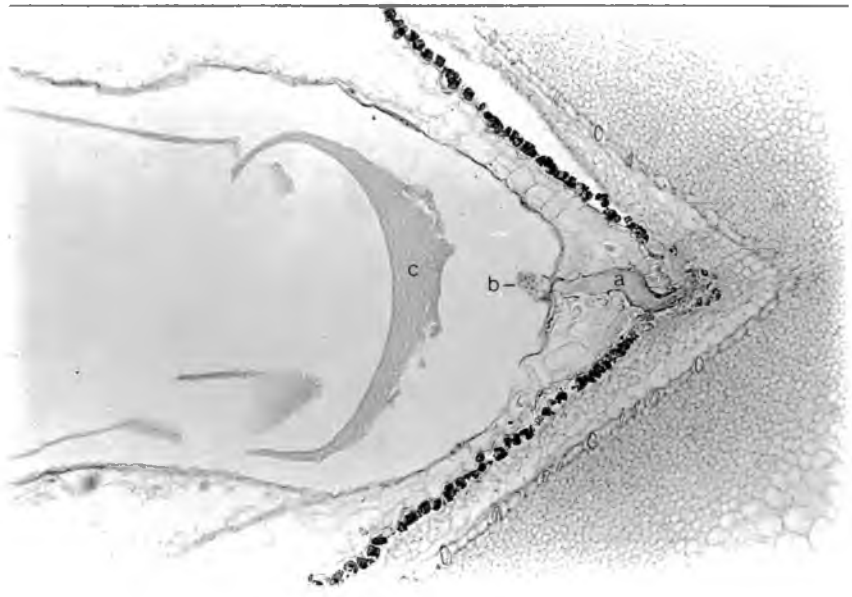

Figure 17

Carica papaya ovule 60 days after intraspecific pollination, with pollen tube (a), embryo (b) and endosperm (c) (X315)

Figure 18

Same section as in Figure 17; whole ovule with endosperm (a) and nucellus (b) (X78)

Figure 19

Carica papaya ovule 75 days after intraspecific pollination, with detail of embryo (a) and pollen tube (b) (X785)

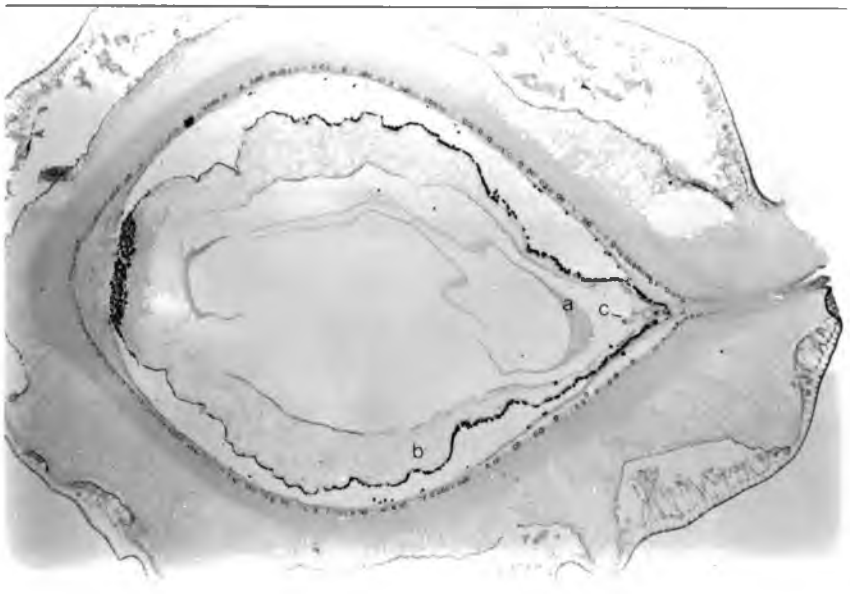

Figure 20

Same section as in Figure 19; whole ovule with endosperm (a), collapsed nucellus (b) and embryo (c) (X78)

suspensor in contact with the tip of an intact pollen tube (Figure 21). The endosperm was becoming cellular, but was only a few cells thick except at the ends (Figure 22). The collapsed nucellus was still present.

The most-developed ovule at 90 days had an embryo with well-defined cotyledons and the beginnings of a vascular system (Figure 23). The suspensor and pollen tube were still evident, although the pollen tube is not included in the illustrations. The embryo was much larger than the one shown in Figure 21, but it was still less than one-tenth the size of a mature embryo. The endosperm was cellular and much thicker than in Figure 21, completely enclosing the embryo and suspensor (Figure 24). However, as with the embryo, it was still only a fraction of the amount seen in mature seeds. The nucellus was reduced to a collapsed remnant.

Most of the ovules at 90 days were at stages of development intermediate to the two extremes shown in Figures 21 and 23.

C. papaya X C. cauliflora: On the 3rd day after pollination of C. papaya with pollen from C. cauliflora, there was no evidence of fertilization, i.e., the synergids were still intact (Figure 25). No pollen tubes were found inside of the micropyles.

On the 9th day, pollen tubes had reached the megagametophyte in most ovules. In some ovules the egg apparatus was still intact and appeared not to have been fertilized as yet; in others (Figure 26), there was a zygote in contact with the pollen tube, accompanied by the remains of the synergids. In one instance, out of approximately

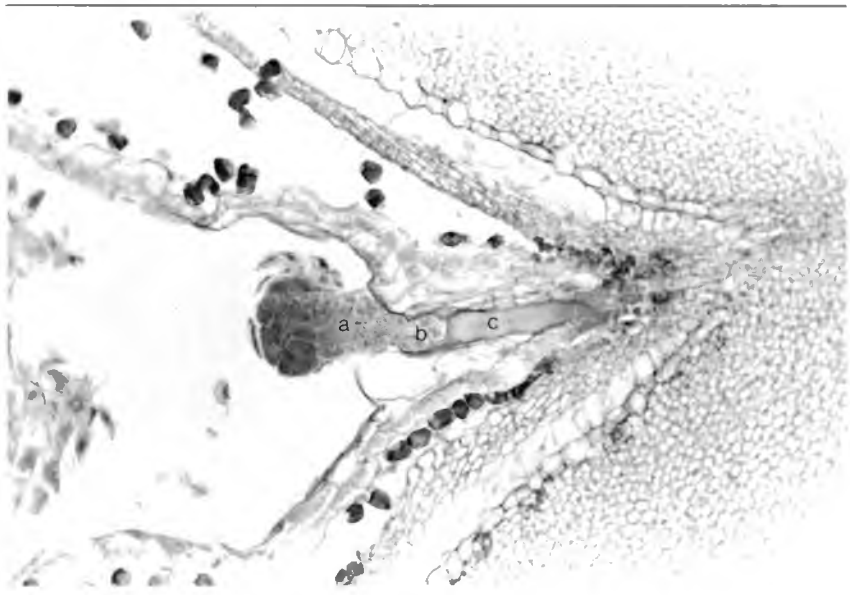

Figure 21

Carica papaya ovule 90 days after intraspecific pollination, with detail of embryo (a), suspensor (b) and pollen tube (c) (X500)

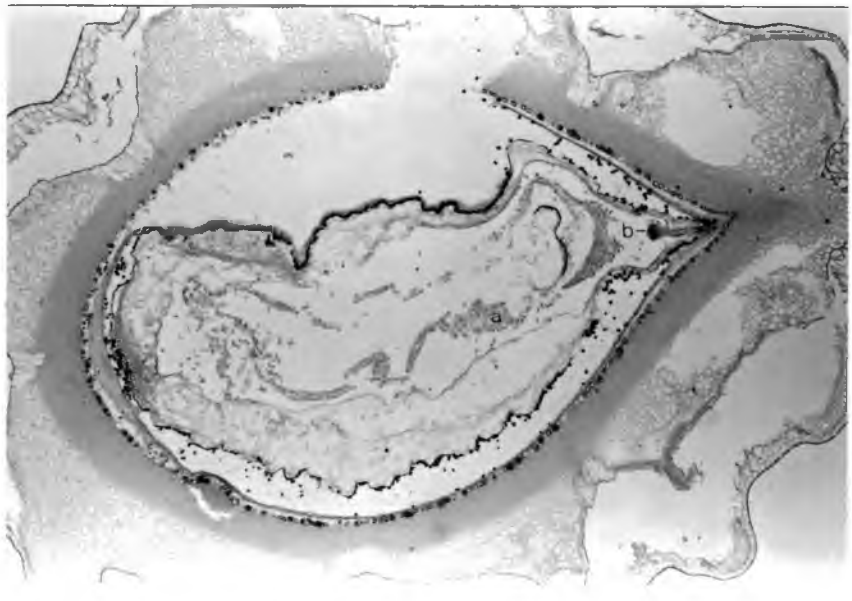

Figure 22

Same section as Figure 21; whole ovule showing relative size of cellular endosperm (a) and embryo (b) (X78)

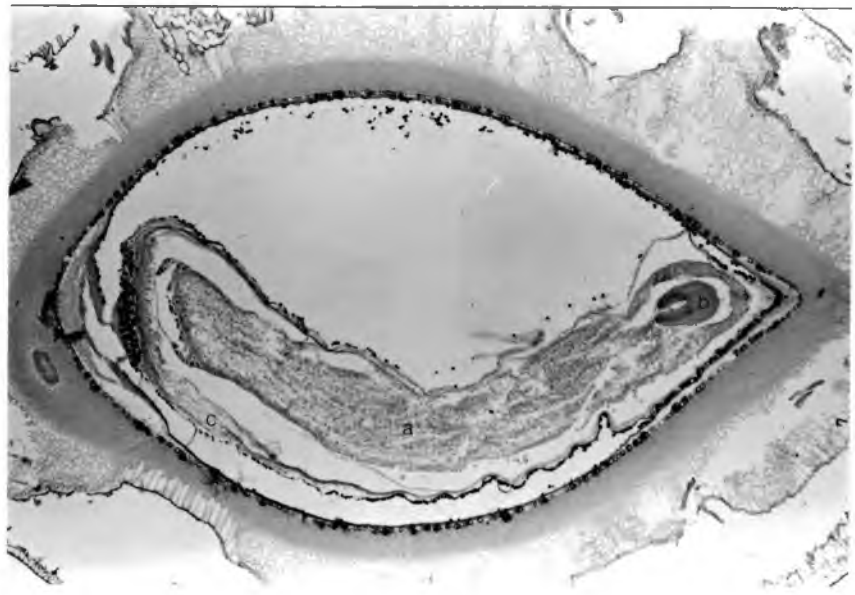

Figure 23

Carica papaya ovule 90 days after intraspecific pollination, whole ovule with cellular endosperm (a), embryo (b) and remnant of nucellus (c) (X78)

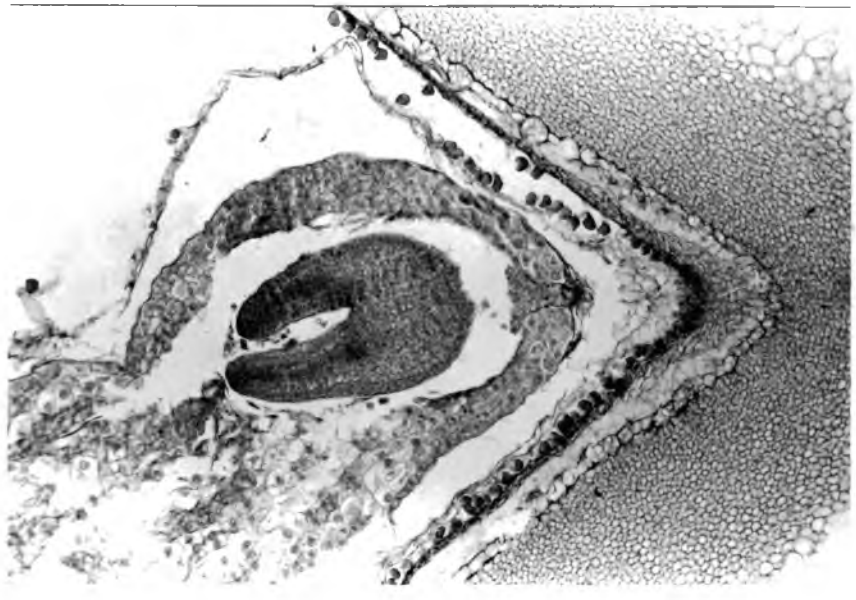

Figure 24

Same section as Figure 23; detail of embryo (X315)

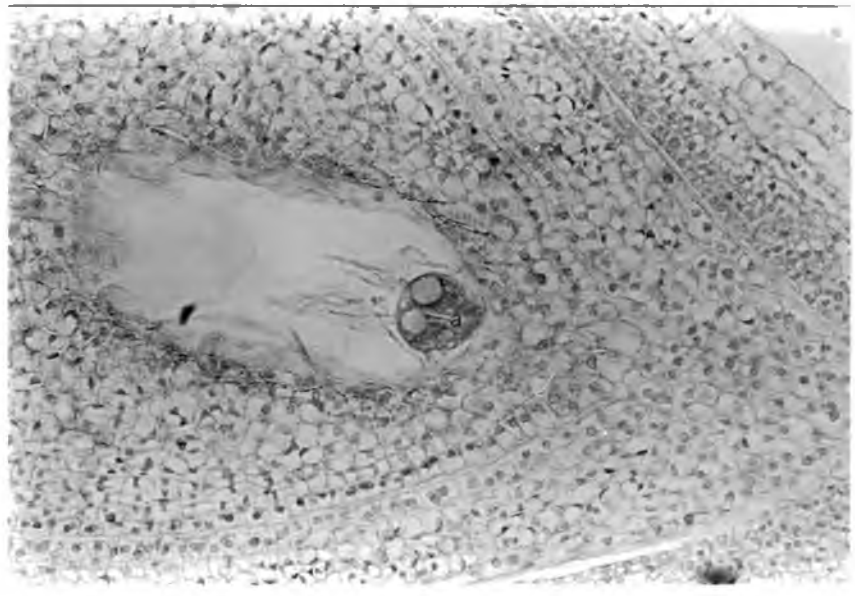

Figure 25

Carica papaya ovule 3 days after pollination by C. cauliflora, with pair of intact synergids (a) (X785)

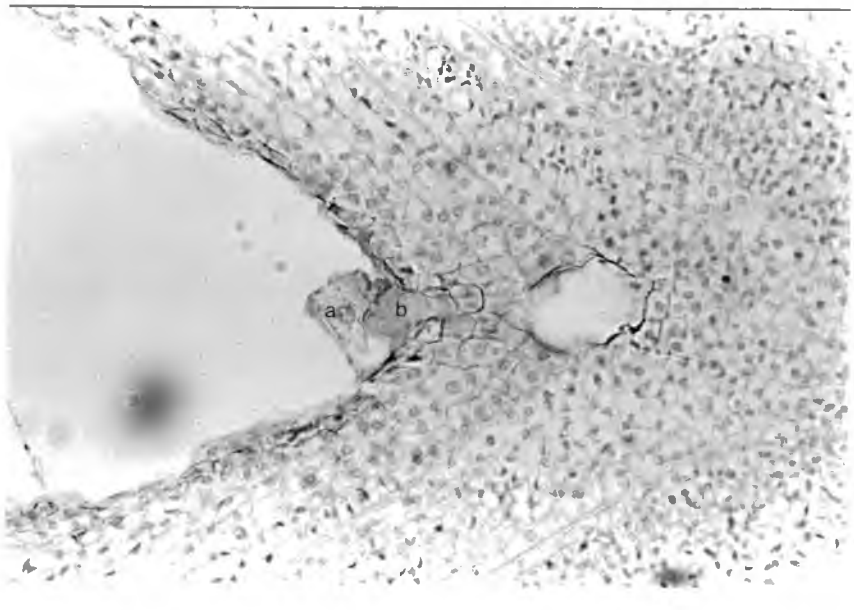

Figure 26

Carica papaya ovule 9 days after pollination by C. cauliflora, with zygote (a) and pollen tube (b) (X785)

20 ovules sectioned, several lightly-stained bodies resembling endosperm nuclei were found (Figure 27).

On the 16th day, the egg apparatus was still evident in most of the ovules. In others the zygote was present (Figure 28). Pollen tubes were not visible in most of the micropyles. The few pollen tubes seen were relatively slender and slightly twisted or sinuous, but not bulbous. No endosperm was found in any of the ovules.

By the 23rd day, the embryo had begun to divide. Figure 29 shows a two-cell pro-embryo in contact with a fairly straight and slender pollen tube. Figure 30 shows an embryo which has undergone several divisions. No endosperm was found in any of the ovules sectioned at this stage. The nucellus was still prominent and considerably larger than at 16 days.

By the 30th day, the embryo had increased to 8 or more cells (Figure 31). The pollen tube was typically narrow, fairly straight, intact and in contact with the embryo. The nucellus was prominent but no endosperm was detected.

On the 45th day, some ovules contained embryos with 10-20 cells. The shape of the embryos varied from somewhat nodular or elongate to irregular in outline. Some embryos appeared to be degenerating. Pollen tubes were intact but did not appear robust. No endosperm was found. The nucellus was mostly intact, with areas of very large, transparent cells. Figure 32 shows an elongated embryo surrounded by nucellus. Figure 33 shows an irregular embryo with a slender pollen tube surrounded by nucellus; the embryo and pollen tube both appear to be degenerating.

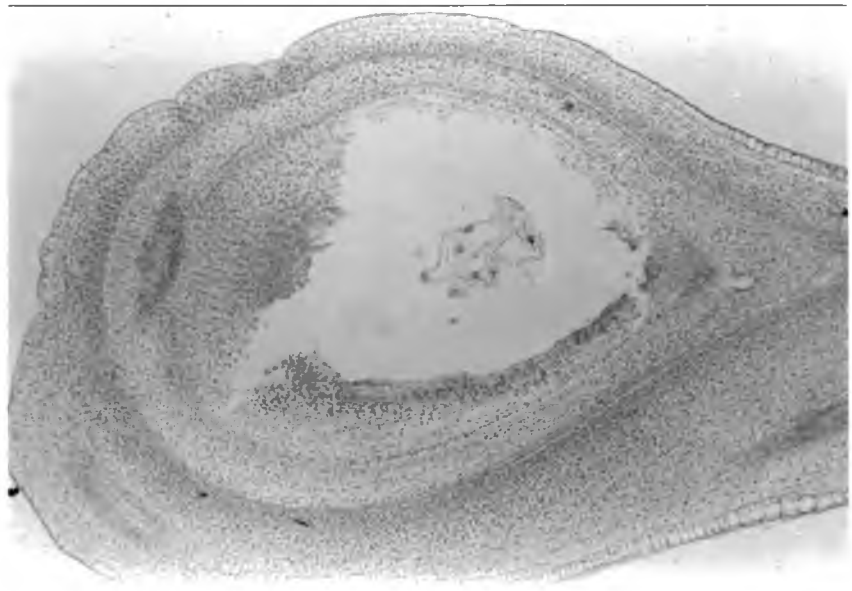

Figure 27

Carica papaya ovule 9 days after pollination by C. cauliflora, with endosperm (X315)

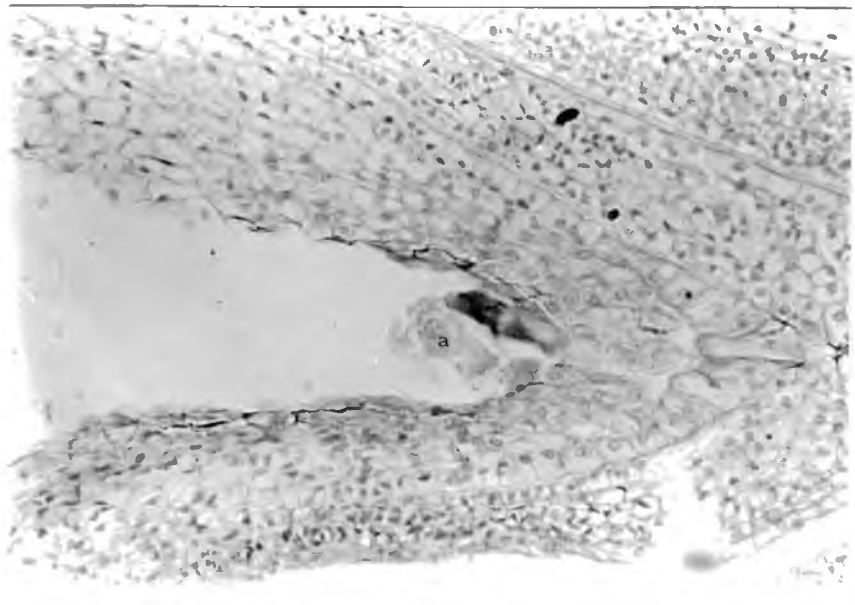

Figure 28

Carica papaya ovule 16 days after pollination by C. cauliflora, with zygote (a) (X785)

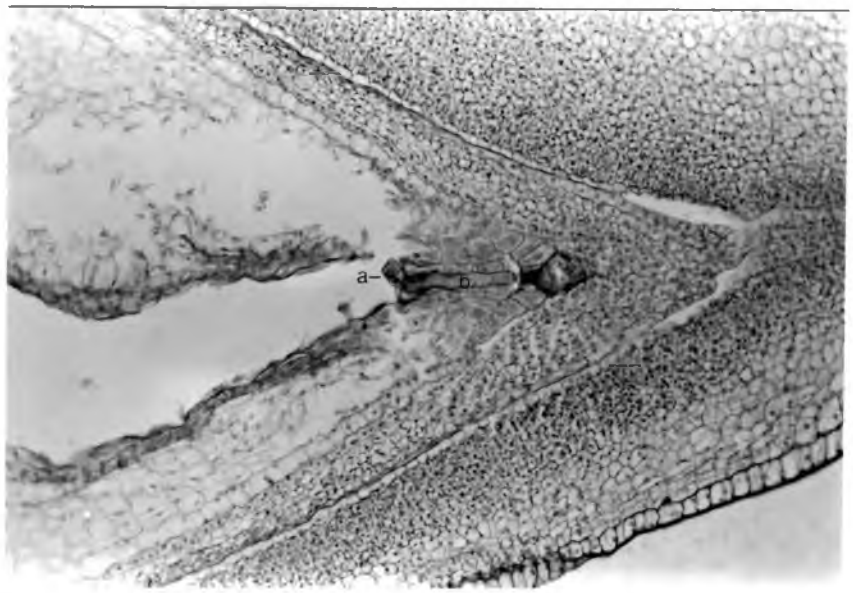

Figure 29

Carica papaya ovule 23 days after pollination by C. cauliflora, with embryo (a) and pollen tube (b) (X500)

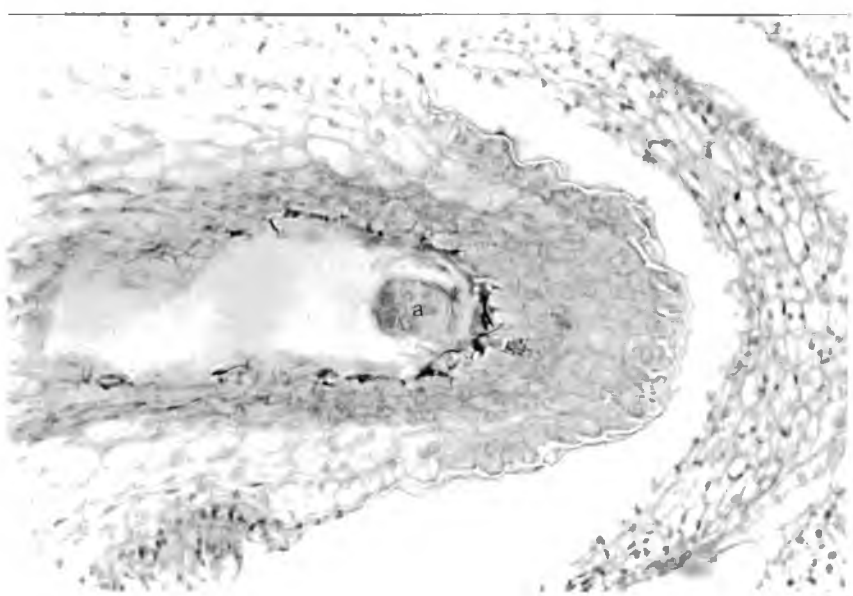

Figure 30

Carica papaya ovule 23 days after pollination by C. cauliflora, with embryo (a) (X785)

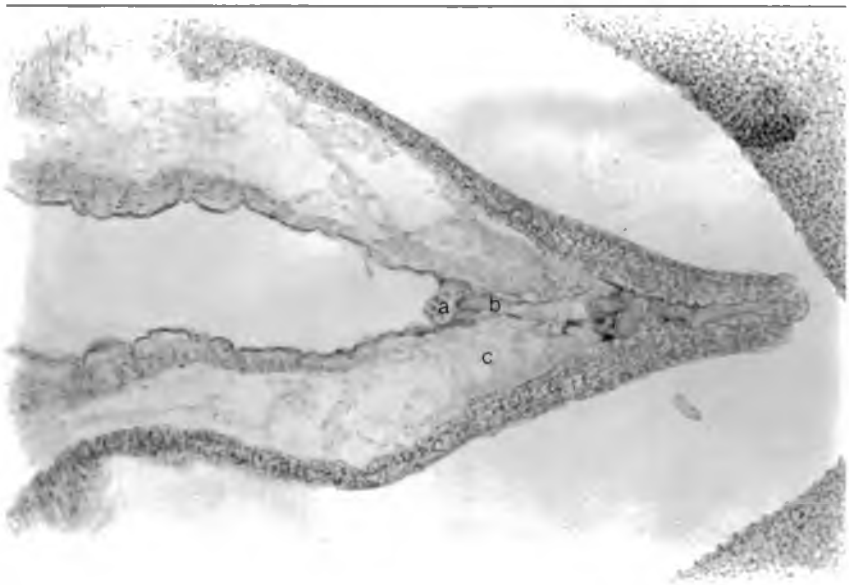

Figure 31

Carica papaya ovule 30 days after pollination by C. cauliflora, with embryo (a), pollen tube (b) and nucellus (c) (X500)

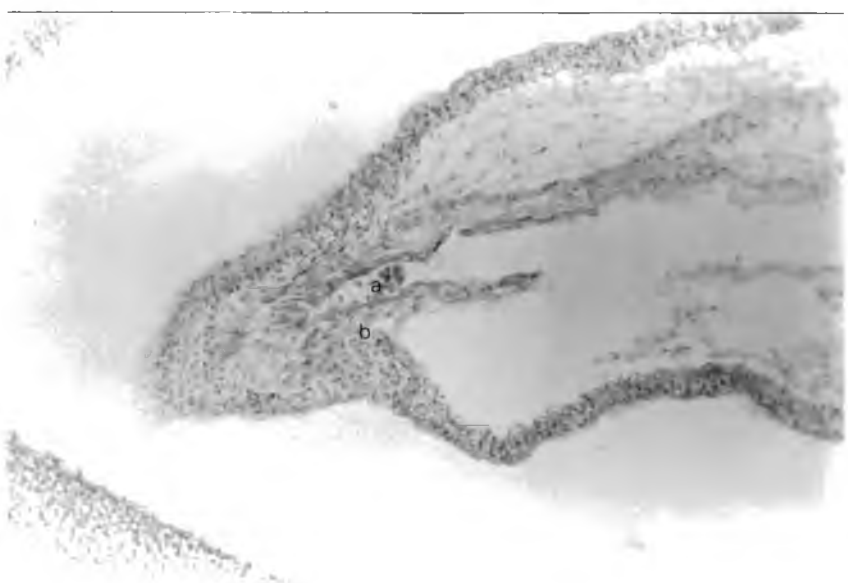

Figure 32

Carica papaya ovule 45 days after pollination by C. cauliflora, with embryo (a) and nucellus (b) (X500)

Figure 33

Carica papaya ovule 45 days after pollination by C. cauliflora, with embryo (a) and pollen tube (b) (X315)

Figure 34

Carica papaya ovule 60 days after pollination by C. cauliflora, with nucellus (a) and abnormal endosperm (b) (X125)

On the 60th day, there was considerable variability among the ovules. Of nine ovules sectioned, one was empty and the remaining eight had small, irregular or nodular embryos of not more than a few dozen cells each. Some of the embryos appeared very weak and may have been degenerating. Pollen tubes could be found, but they appeared to be weakening or breaking down. In some ovules the entire interior structure had collapsed inside of the integuments; in others the nucellus was still prominent but appeared very spongy. Two ovules had a peculiar layer where the endosperm would normally be found (Figure 34). This layer contained several bodies somewhat resembling nuclei. The ovule depicted also had a large, intact nucellus. Figure 35 shows an ovule with a collapsed interior. The embryo is elongated and the pollen tube appears to have degenerated. Figure 36 shows a small embryo in contact with an apparently intact, but rather weak-looking, pollen tube. Figure 37 shows an embryo with a very irregular outline surrounded by a relatively prominent nucellus.

On the 75th day, seven of the nine ovules sectioned had intact embryos that were larger than the 60 day material, but were nodular or irregular in outline and undifferentiated. Another ovule contained a clearly degenerating embryo: the entire embryo had collapsed so much that all of it was included on a single 11 micrometer section (Figure 38), whereas other embryos would appear over several sections. These eight ovules had varying degrees of collapse within the integuments, and the pollen tubes had also collapsed. The remaining ovule had a larger undifferentiated embryo with well over 100 cells and an intact pollen tube in contact with it; the embryo is best seen in

Figure 35

Carica papaya ovule 60 days after pollination by C. cauliflora, with collapsed interior, irregular embryo (a) and deteriorating pollen tube (b) (X315)

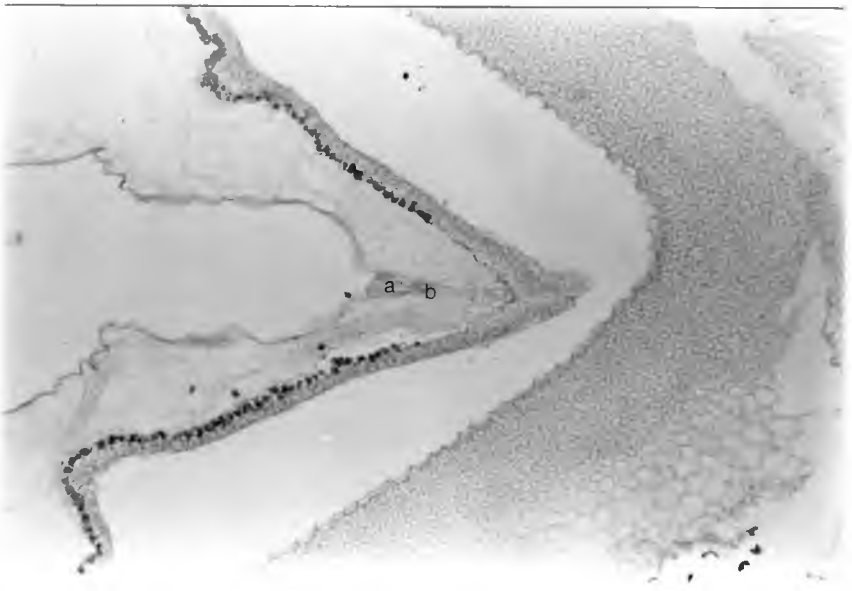

Figure 36

Carica papaya ovule 60 days after pollination by C. cauliflora, with embryo (a) and pollen tube (b) (X315)

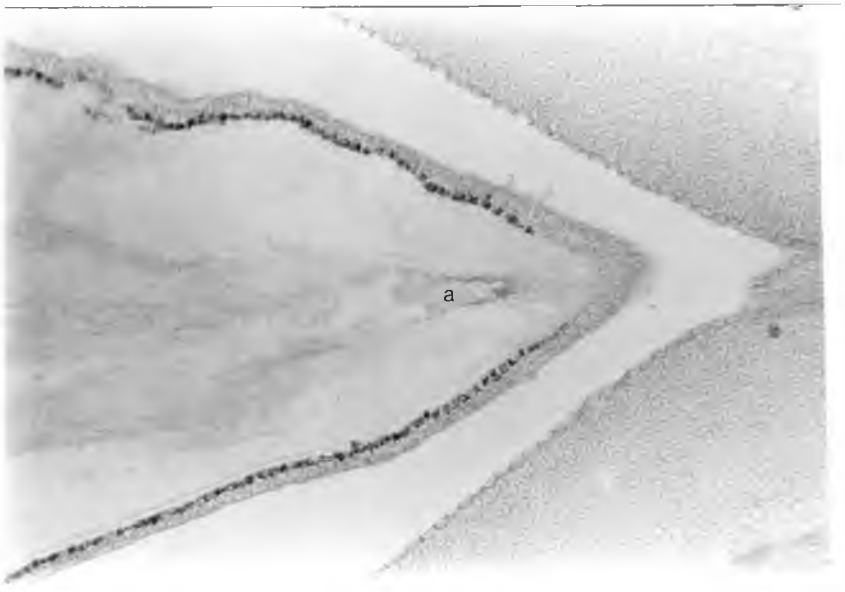

Figure 37

Carica papaya ovule 60 days after pollination by C. cauliflora, with irregular embryo (a) (X315)

Figure 38

Carica papaya ovule 75 days after pollination by C. cauliflora, with degenerating embryo (a) and pollen tube (b) (X315)

one section (Figure 39), while the pollen tube is more visible in an adjacent section (Figure 40). Most of the 75-day ovules had fairly intact nucelli.

On the 90th day, two of the nine ovules sectioned contained clearly aborted embryos. One of these embryos (Figure 41) was very elongated with one half of its length completely withered. The remaining seven ovules contained embryos larger than those seen at 75 days, but none were differentiated and most of the ovules had collapsed within the integuments. Pollen tubes could still be seen but were degenerating, judging by their somewhat crushed appearance. The nucellus had collapsed in all of the ovules. Figure 42 shows an ovule with a collapsed embryo sac and a declining embryo. Figure 43 shows a severely collapsed embryo sac enclosing an irregular embryo and the remains of the pollen tube; a portion of the integuments is visible at the lower right. Only one ovule showed any evidence of endosperm (Figure 44), and this was very abnormal and underdeveloped, with greatly enlarged nuclei.

Dissection of over five hundred fresh seeds of the C. papaya X C. cauliflora cross, ranging in age from 76 days to 6 months (ripe fruit), revealed no seeds with either an embryo or endosperm that could be seen under low magnification on the dissection microscope.

Intraspecific C. cauliflora: Results of intraspecific mating of C. cauliflora were as follows: On the 3rd day after pollination no pollen tubes could be found entering the micropyles of the ovules. Figure 45 shows a section through an open micropyle.

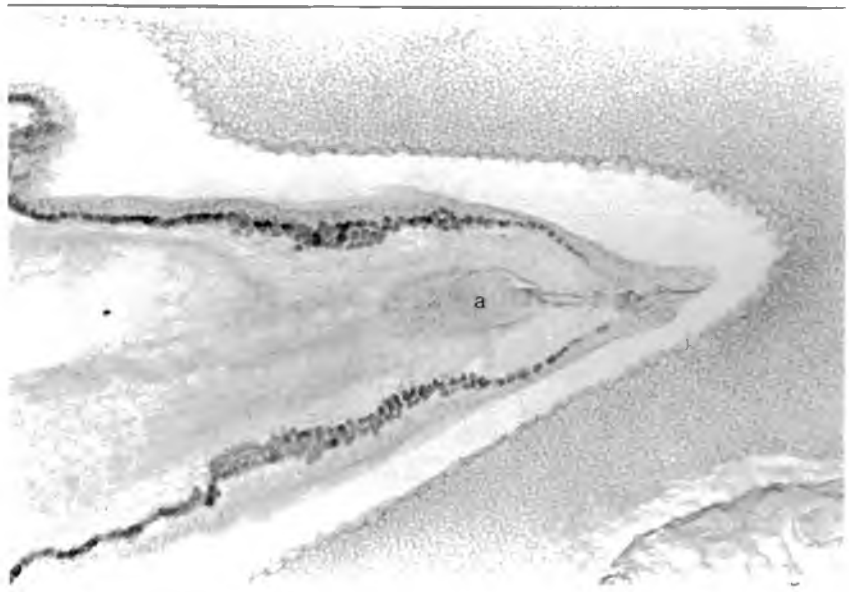

Figure 39

Carica papaya ovule 75 days after pollination by C. cauliflora, with embryo (a) (X315)

Figure 40

Same ovule as Figure 39; adjacent section including pollen tube (a) (X315)

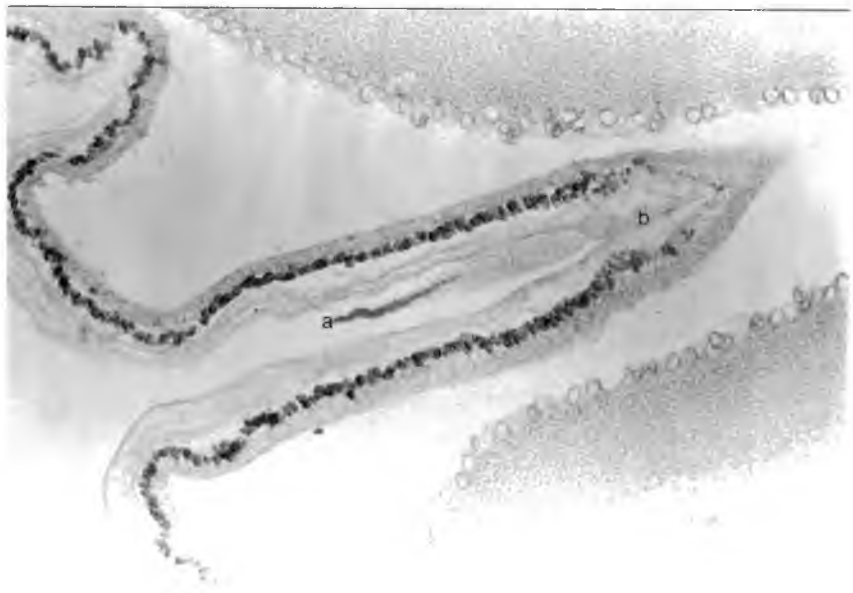

Figure 41

Carica papaya ovule 90 days after pollination by C. cauliflora, showing embryo with necrotic tip (a) and deteriorated pollen tube (b) (X315)

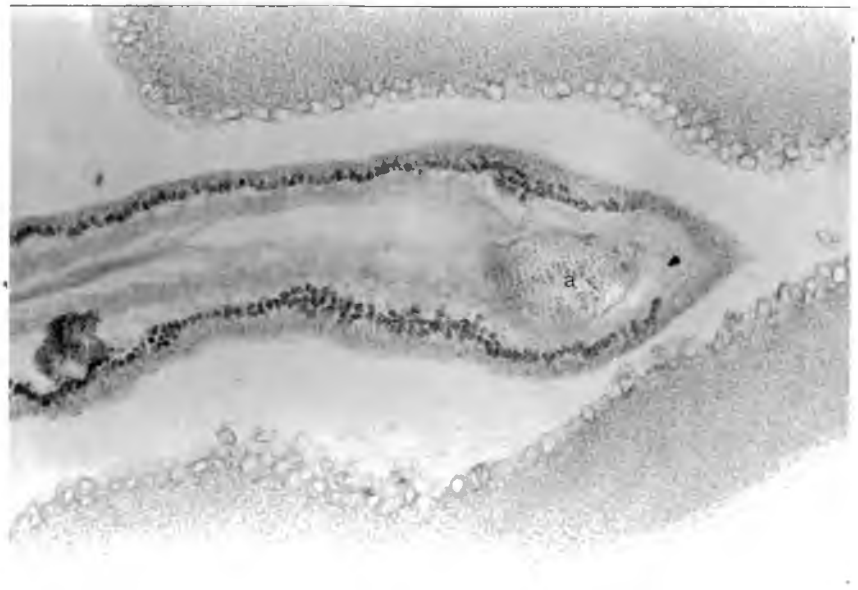

Figure 42

Carica papaya ovule 90 days after pollination by C. cauliflora, with embryo (a) and collapsed interior (X315)

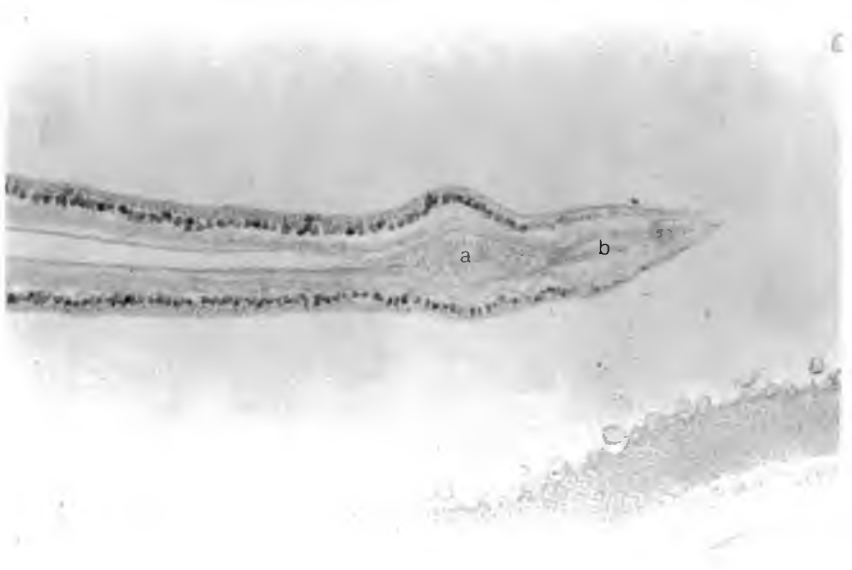

Figure 43

Carica papaya ovule 90 days after pollination by C. cauliflora, with collapsed interior, degenerating embryo (a) and deteriorating pollen tube (b) (X315)

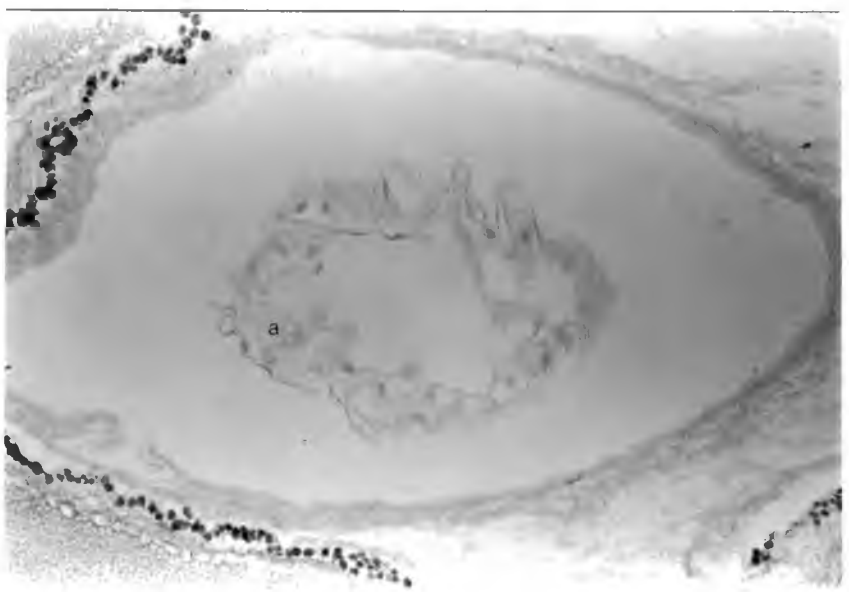

Figure 44

Carica papaya ovule 90 days after pollination by C. cauliflora, oblique section of abnormal endosperm (a) with enlarged nuclei (X315)

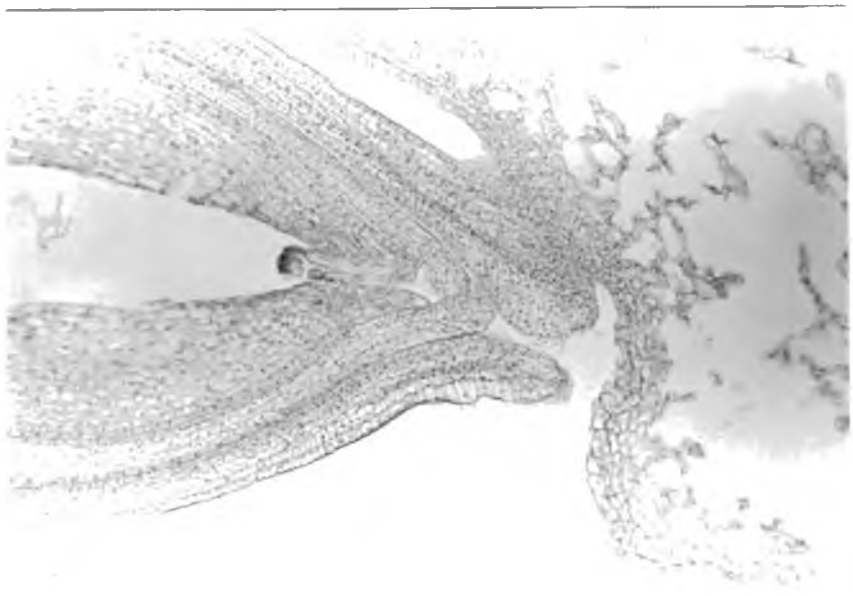

Figure 45

Carica cauliflora ovule 3 days after intraspecific pollination; no pollen tube in micropyle (X315)

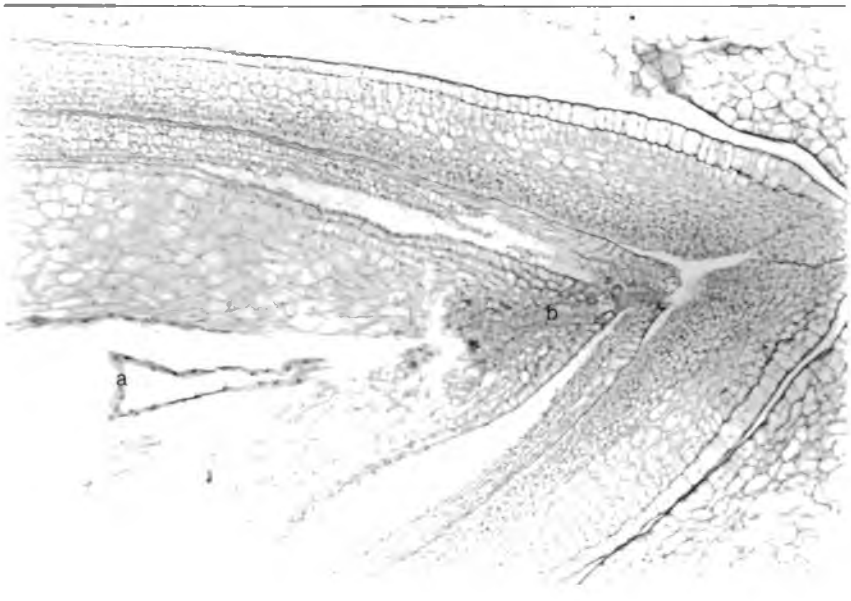

Figure 46

Carica cauliflora ovule 16 days after intraspecific pollination, with multi-nucleate endosperm (a) and pollen tube (b) (X315)

On the 9th day, there were still no pollen tubes found entering the ovules.

By the 16th day, double fertilization had occurred; the synergids had collapsed and the ovules contained zygotes and free-nuclear endosperm with many nuclei. Figure 46 shows a section with a portion of the pollen tube and the endosperm visible.

On the 23rd day, the zygote had divided to form a two-cell pro-embryo (Figure 47). The endosperm layer had thickened and the nucellar cells were very large and transparent (Figure 48).

On the 30th day, an embryo consisting of about eight cells had developed (Figure 49). The pollen tube persisted and was in contact with the embryo. The nucellus had partially collapsed. A view of the entire ovule (Figure 50) shows the collapsing nucellus and the endosperm, which had increased considerably since day 23. A close-up view of the endosperm at the chalazal end indicates that it was still free nuclear (Figure 51).

By the 45th day, the embryo contained a few dozen cells and was becoming club-shaped with a suspensor (Figure 52). The pollen tube was still intact, and the collapsed nucellus was still visible. The ovule had increased considerably in size (Figure 53),and the endosperm was slightly thicker than at day 30. Details of the endosperm at the mid-section of the ovule (Figure 54) show that it was still free nuclear.

On the 62nd day, a rapid spurt of development was evident. The embryo had differentiated to the heart-shaped stage and was approaching macroscopic proportions (Figure 55). The pollen tube was still intact and in contact with the embryo. The endosperm had become cellular and

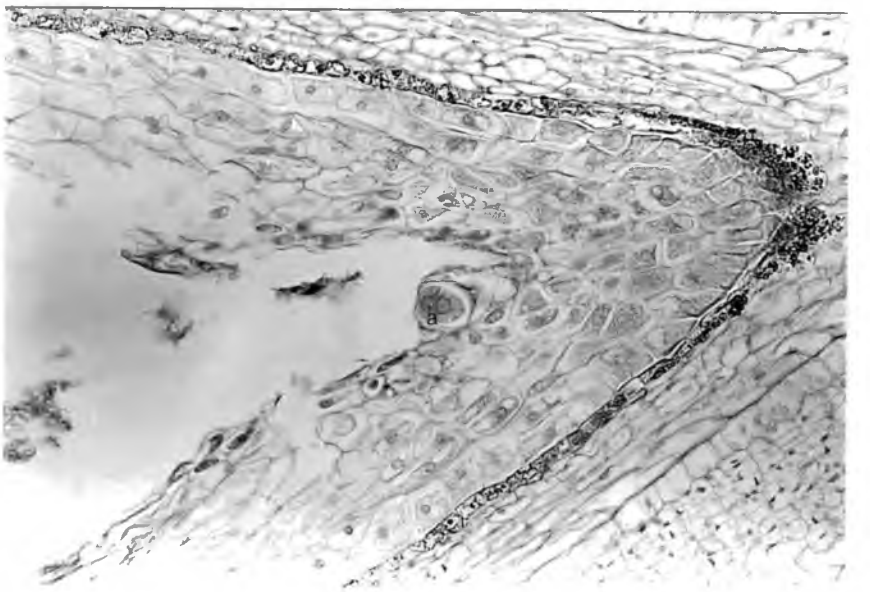

Figure 47

Carica cauliflora ovule 23 days after intraspecific pollination, with two-cell embryo (a) (X785)

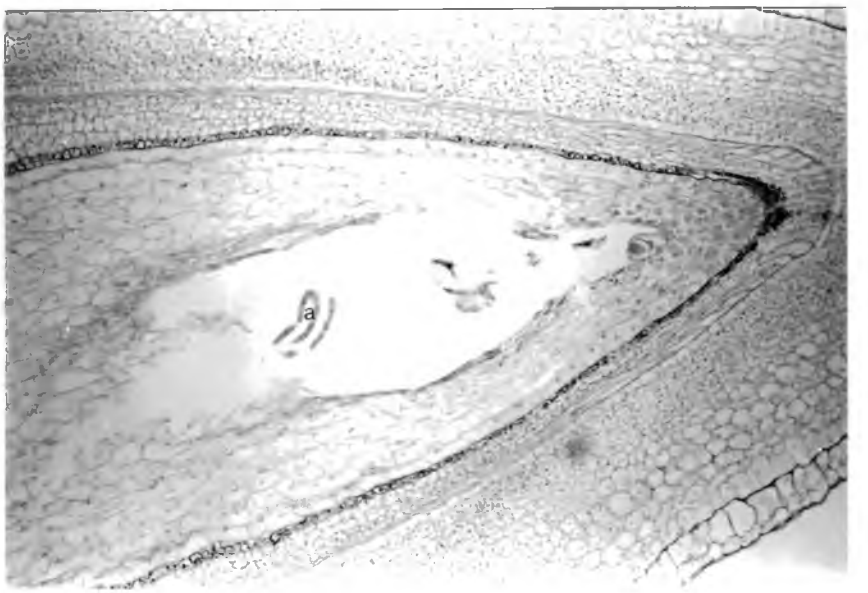

Figure 48

Same section as Figure 47; fragments of endosperm visible (a) (X315)

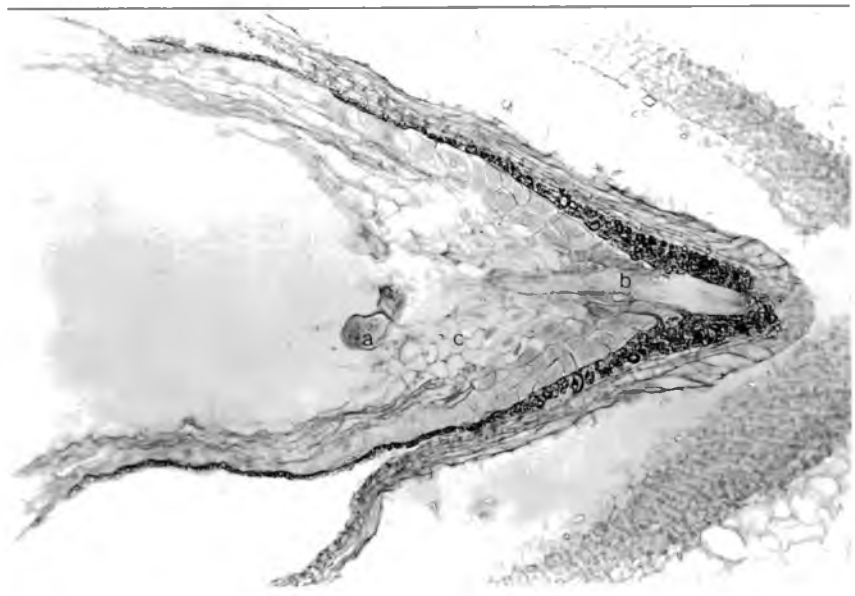

Figure 49

Carica cauliflora ovule 30 days after intraspecific pollination, with embryo (a), part of pollen tube (b) and collapsed nucellus (c) (X500)

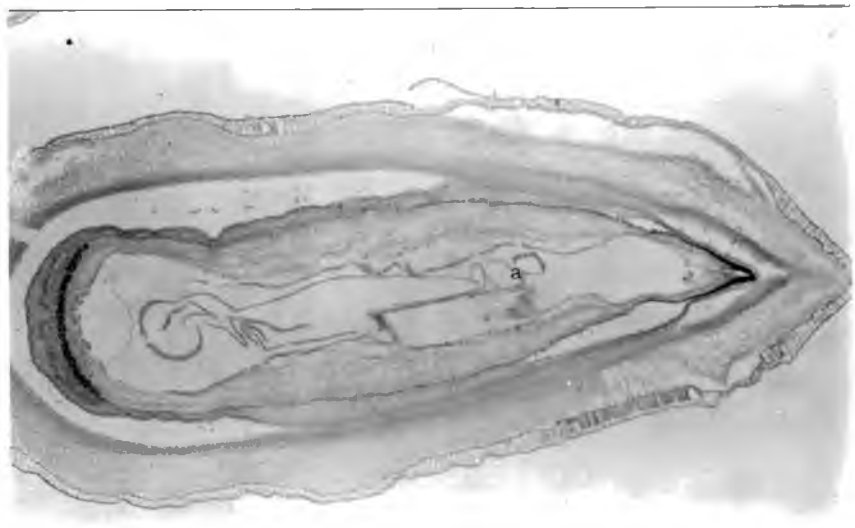

Figure 50

Carica cauliflora ovule 30 days after intraspecific pollination, same section as Figure 49; entire ovule with endosperm (a) (X78)

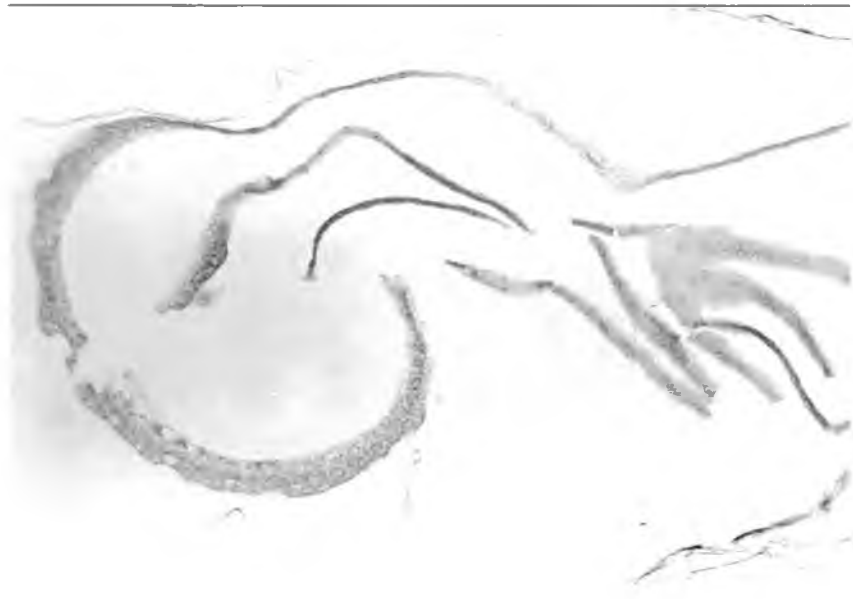

Figure 51

Carica cauliflora ovule 30 days after intraspecific pollination, same section as Figures 49-50; detail of endosperm (X500)

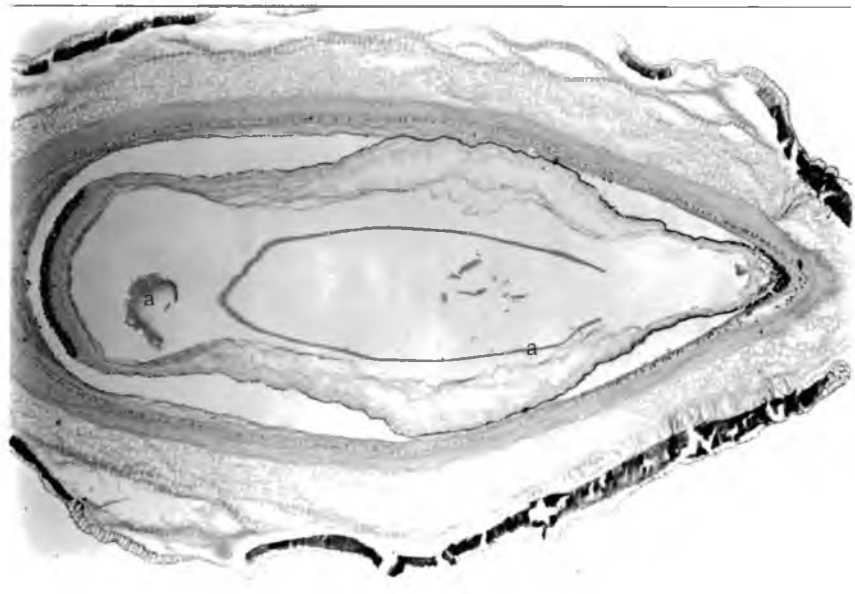

Figure 52

Carica cauliflora ovule 45 days after intraspecific pollination, entire ovule with endosperm (a) (X78)

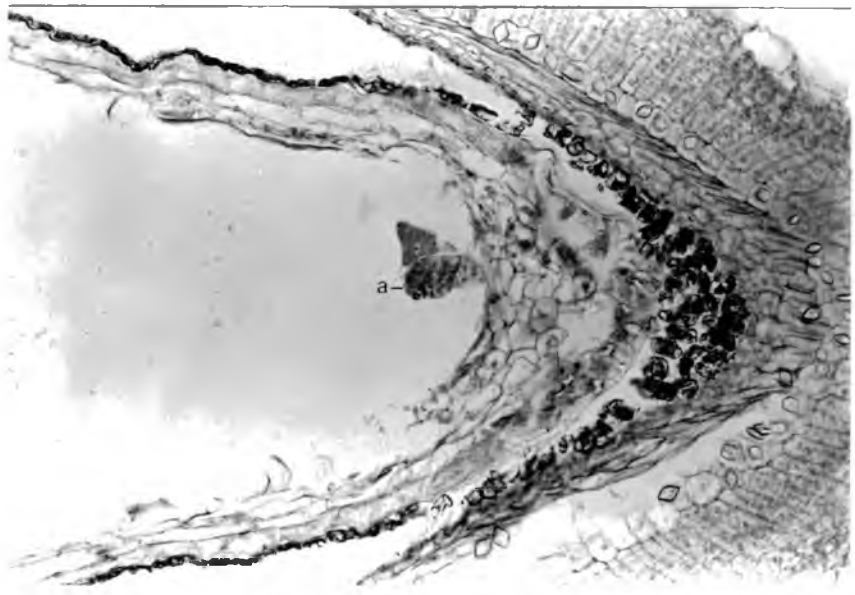

Figure 53

Carica cauliflora ovule 45 days after intraspecific pollination, same section as in Figure 52, showing embryo (a) with piece of endosperm attached (X500)

Figure 54

Same section as in Figures 52-53; detail of endosperm layer (X500)

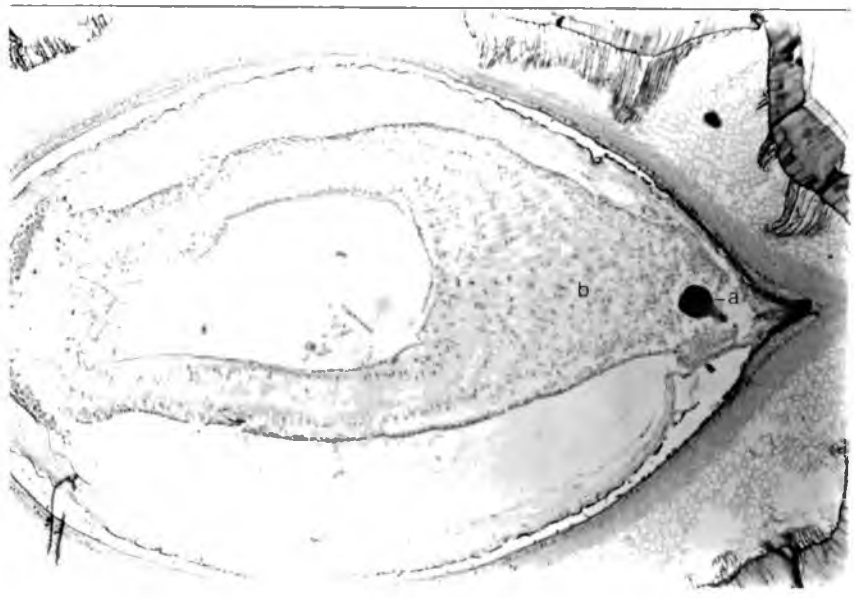

Figure 55

Carica cauliflora ovule 62 days after intraspecific pollination, with embryo (a) and cellular endosperm (b) (X78)

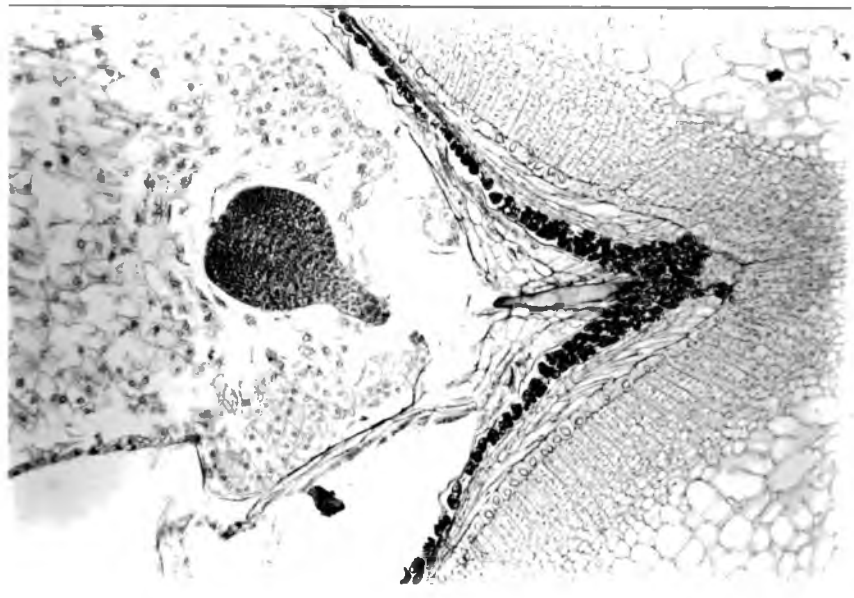

Figure 56

Same section as in Figure 55; detail of embryo surrounded by cellular endosperm; pollen tube also visible (X315)

was about two-thirds of its mature size, completely surrounding the embryo (Figure 56).

By the 75th day, the embryo was becoming vascularized and the cotyledon primordia had begun to elongate (Figure 57). The endosperm was nearly full sized but not especially dense. The remains of the nucellus were nearly obliterated by the expanding endosperm. The pollen tube was still intact and in contact with the embryo, although they are separated in the illustration provided (Figure 58) due to shrinkage of the specimen during fixation.

By the 90th day, the increase in development was dramatic. The embryo had reached three-quarters mature size and had assumed its mature form with cotyledons, root and shoot meristems and well-developed vascular system (Figure 59). The endosperm had filled in and appeared much denser. The nucellus had been obliterated. The suspensor was present at the end of the radicle and in close proximity to the still-intact pollen tube (Figure 60).

C. cauliflora X C. papaya: Pollination of C. cauliflora with pollen from C. papaya gave the following results:

On the 3rd day, there was no evidence of fertilization or penetration of the micropyles by pollen tubes. Mature megagametophytes with intact egg apparatuses could be seen. Figure 61 shows a section with both synergids and polar nuclei visible. Figure 62 shows a section from a different ovule with one synergid, one polar nucleus and the egg visible.

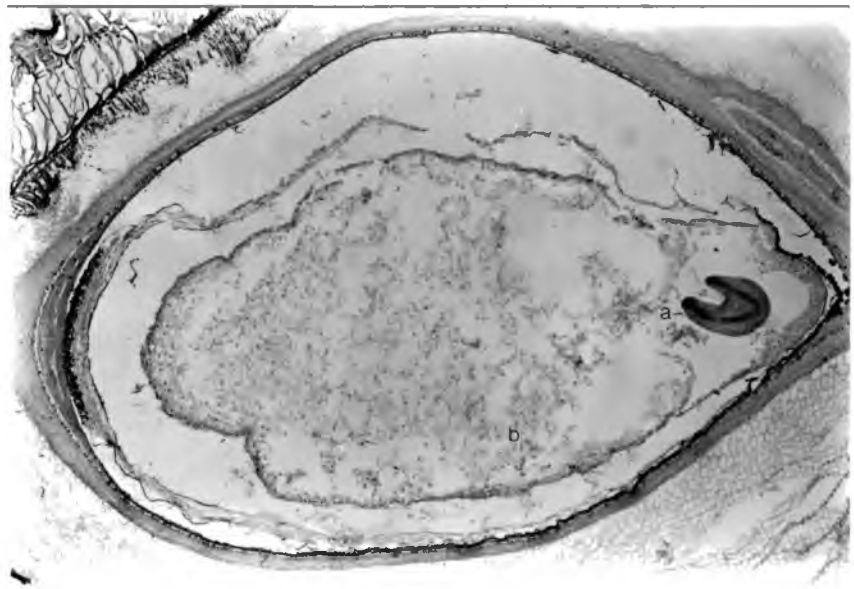

Figure 57

Carica cauliflora ovule 75 days after intraspecific pollination; entire ovule showing embryo (a) and cellular endosperm (b) (X78)

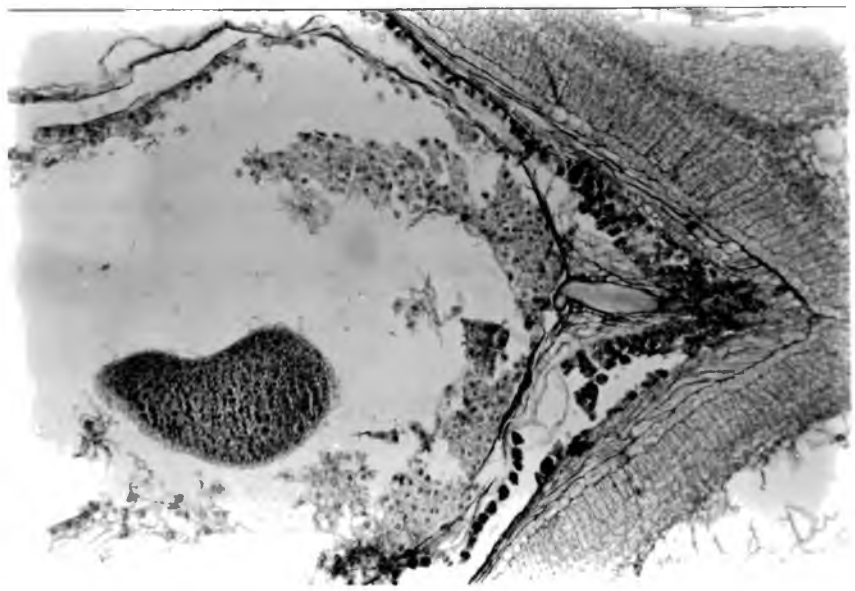

Figure 58

Same ovule as in Figure 57; different section including pollen tube (X315)

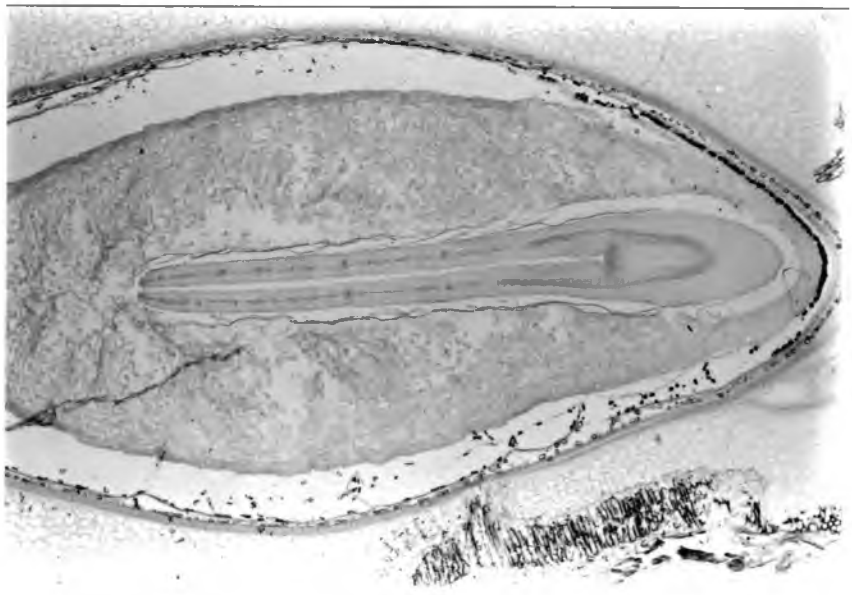

Figure 59

Carica cauliflora ovule 90 days after intraspecific pollination, with advanced embryo and endosperm (X78)

Figure 60

Same ovule as in Figure 59; different section including suspensor (a) extending from radicle, and pollen tube (b) (X315)

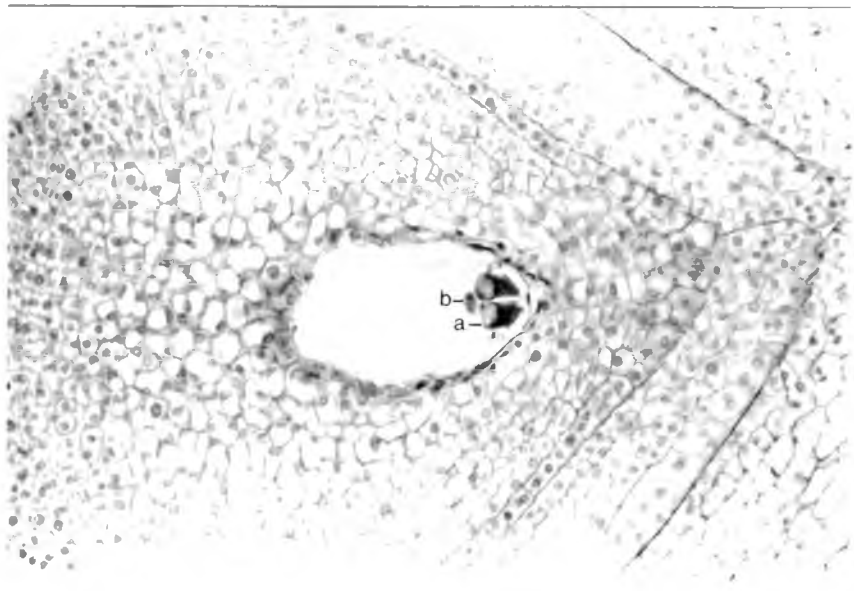

Figure 61

Carica cauliflora ovule 3 days after pollination by C. papaya, with unfertilized synergids (a) and polar nuclei (b) (X785)

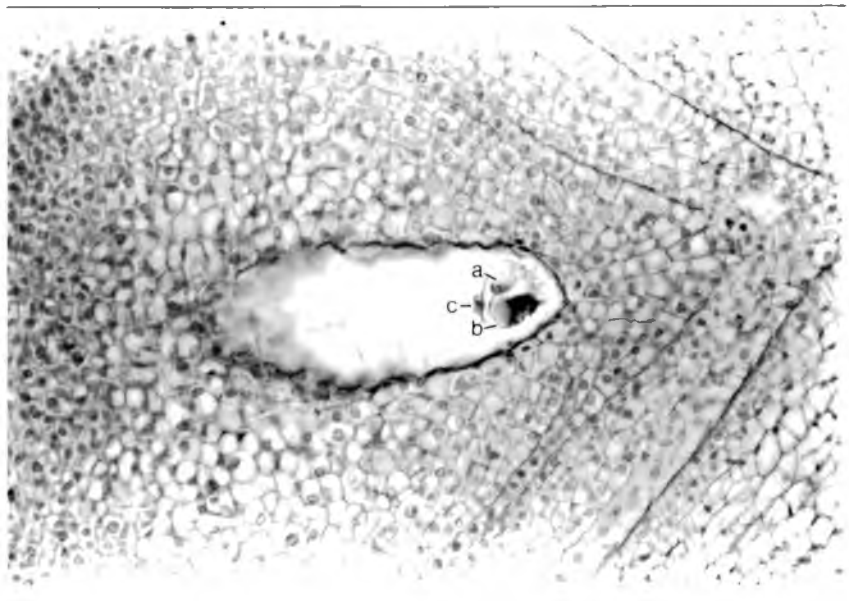

Figure 62

Carica cauliflora ovule 3 days after pollination by C. papaya, with egg (a), one synergid (b) and one polar nucleus (c) (X785)

On the 9th day, pollen tubes were found in the micropyles, but it could not be determined whether fertilization had occurred. Figure 63 shows an ovule and a portion of the ovary wall with a pollen tube visible outside of the ovule and extending into the micropyle. The same pollen tube is also visible farther in the micropyle (Figure 64).

By the 14th day, the pollen tubes had reached the megagametophyte. The end of the pollen tube in the nucellar beak was fairly massive (Figure 65), compared with the same pollen tube, at the same magnification, extending through the inner integument (Figure 66). No intact egg apparatus could be found, but neither could any zygotes be identified with certainty, nor was any endosperm observed. Consequently, it was not possible to determine whether or not fertilization had occurred in the more than ten ovules included in the piece of ovary sectioned.

By the 22nd day, four-cell pro-embryos could be found. No endosperm was observed. The nucellus was intact and had enlarged. Figure 67 shows a slightly oblique cross section through an ovule with the four-cell embryo visible in the center.

By the 28th day, variability was apparent among the twelve ovules sectioned. Ten ovules showed no increase in the embryo compared to the 22-day material. The remaining two ovules had embryos of at least sixteen cells. No endosperm was detected in any of the ovules. Pollen tubes were intact. The nucellus had expanded and was composed of large, transparent cells. Figure 68 shows one of the larger embryos detected; also visible are a sliver of the pollen tube and a portion of the nucellus.

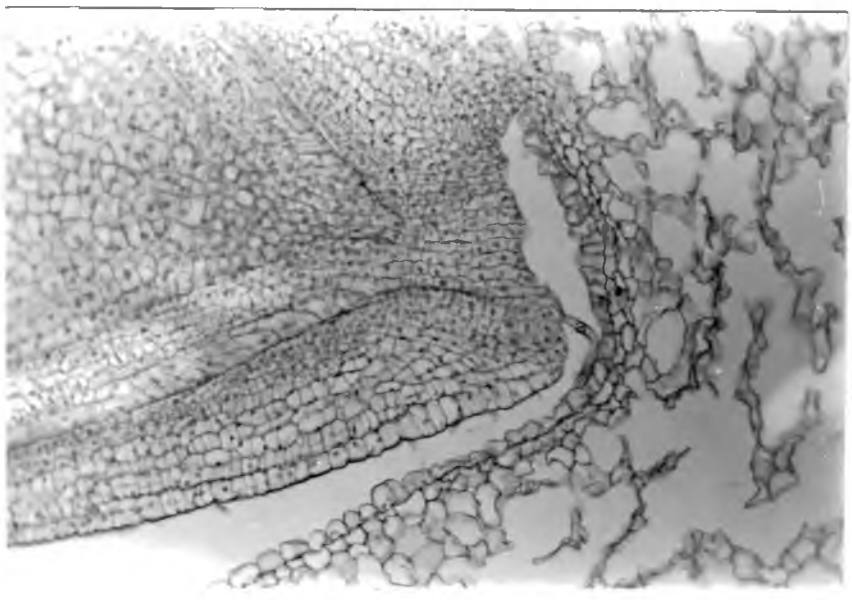

Figure 63

Carica cauliflora ovule 9 days after pollination by C. papaya, with pollen tube (a) entering micropyle (X500)

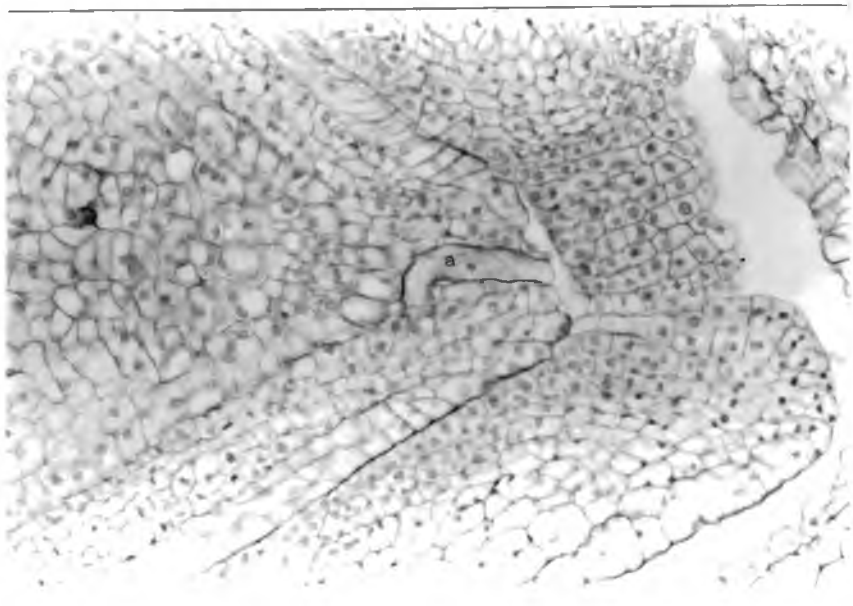

Figure 64

Same ovule as in Figure 63; different section with pollen tube (a) inside of micropyle (X785)

Figure 65

Carica cauliflora ovule 14 days after pollination by C. papaya, with pollen tube (a) in micropyle (X785)

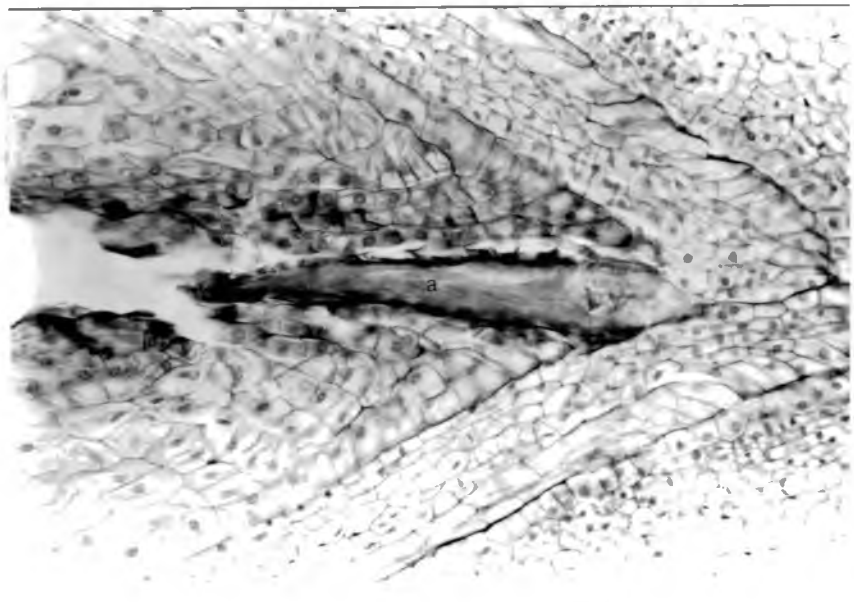

Figure 66

Same ovule as in Figure 65; different section with end of pollen tube (a) extending to egg sac (X785)

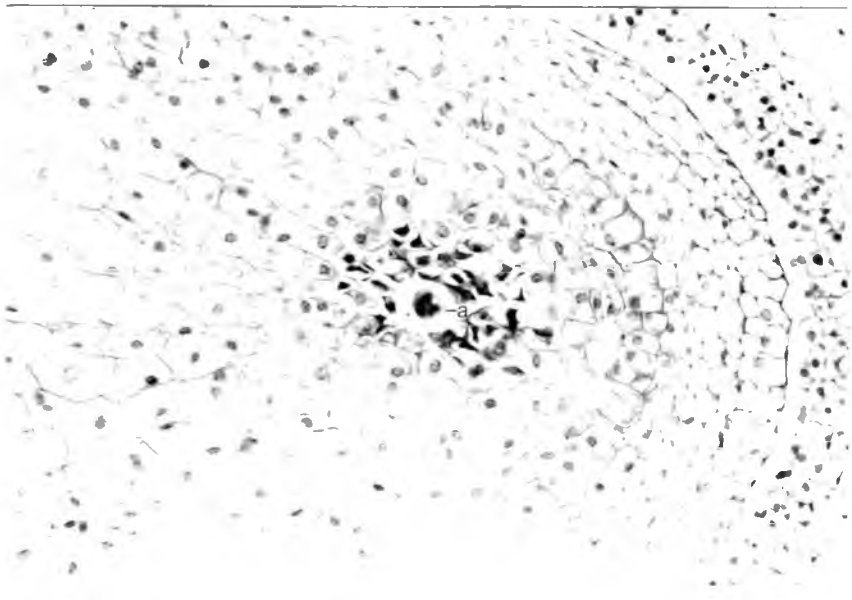

Figure 67

Carica cauliflora ovule 22 days after pollination by C. papaya, cross section with pro-embryo (a) (X785)

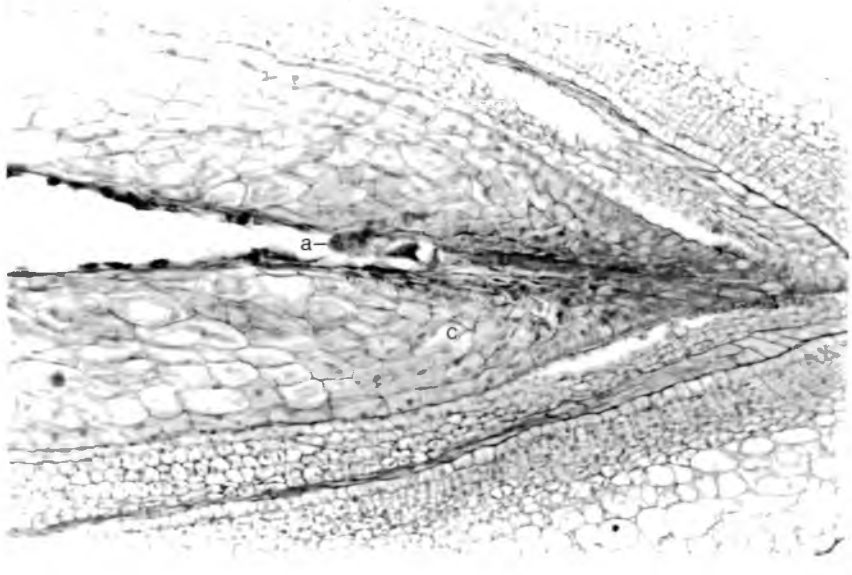

Figure 68

Carica cauliflora ovule 28 days after pollination by C. papaya, with embryo (a), pollen tube (b) and nucellus (c) (X500)

On the 44th day, only one of the twelve ovules sectioned appeared to have a developing embryo. The embryo contained about two dozen cells and was oblong but undifferentiated (Figure 69). No endosperm was observed in any of the ovules. Pollen tubes were present in all of the ovules and appeared to be intact, even though the majority of the ovules were collapsing inside of the integuments. The nucellar cells were greatly enlarged and quite transparent.

On the 62nd day, most of the ovules were smaller than those produced by intraspecific pollinations and had flattened sides. Pollen tubes were still visible and relatively intact: two sections from one ovule trace the pollen tube through the nucellus (Figure 70) and in contact with the embryo (Figure 71). Nucellar cells in all of the ovules were collapsed except near the micropyle. No endosperm was observed. Eight of the twelve ovules sectioned had developing embryos of varying size and degree of differentiation. All of the embryos were highly abnormal and characterized by a tendency to divide or bud into clusters of poorly differentiated growth. At least three different types of embryos could be identified. The first type (Figure 72) was generally smaller than the others, consisting of an irregular mass of embryonic tissue, which in Figure 72 appeared to be degenerating at the chalazal end. The second type (Figure 73) was larger than the first, also irregular, and apparently embryogenic, budding off small masses with no obvious differentiation in individual lobes. The third type (Figures 71 and 74) was clearly embryogenic, producing multiple embryos attached in a common cluster at the micropylar end. The individual

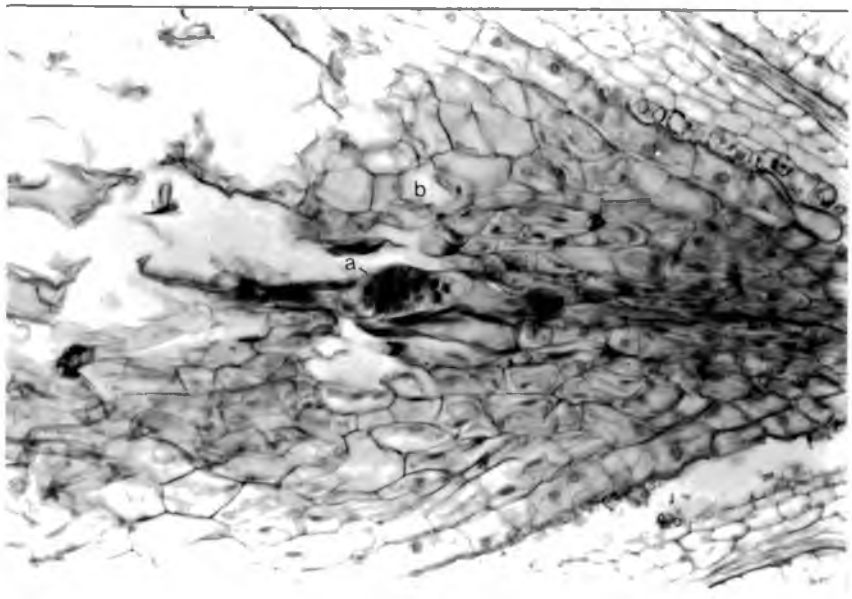

Figure 69

Carica cauliflora ovule 44 days after pollination by C. papaya, with embryo (a) and nucellus (b) (X785)

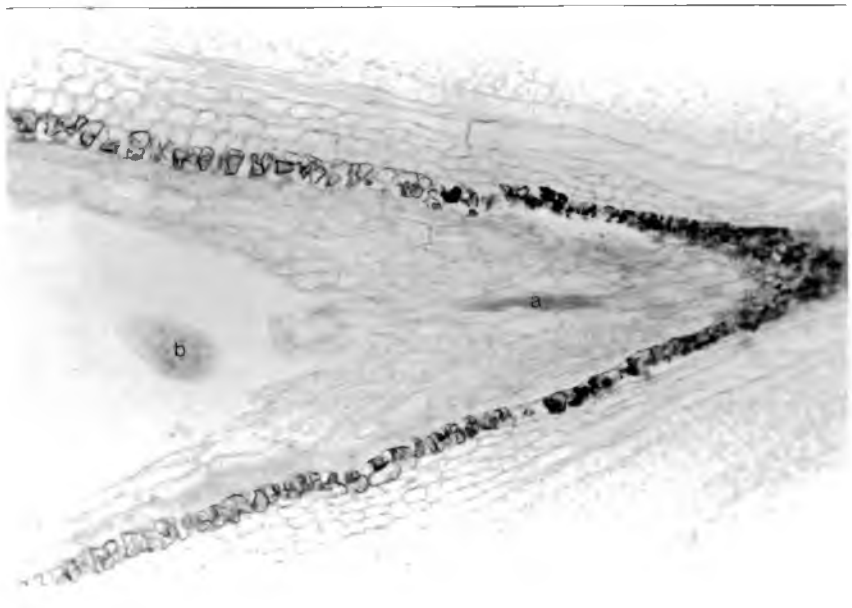

Figure 70

Carica cauliflora ovule 62 days after pollination by C. papaya, with pollen tube (a) in nucellus; portion of embryo also visible (b) (X500)

Figure 71

Carica cauliflora ovule 62 days after pollination by C. papaya, same ovule as in Figure 70; adjacent section with pollen tube in contact with embryo (a) (X500)

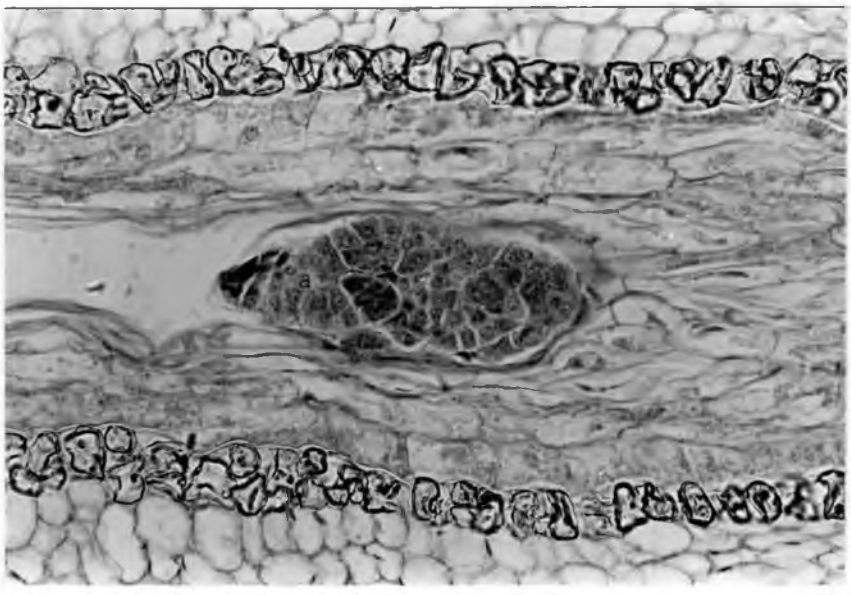

Figure 72

Carica cauliflora ovule 62 days after pollination by C. papaya, containing undifferentiated embryo with necrotic tip (a) (X785)

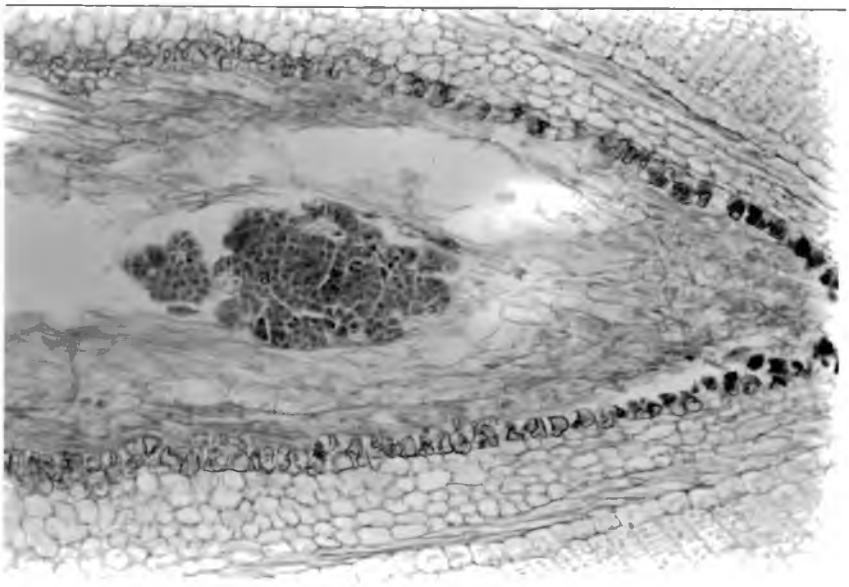

Figure 73

Carica cauliflora ovule 62 days after pollination by C. papapa, with undifferentiated embryogenic tissue (a) (X500)

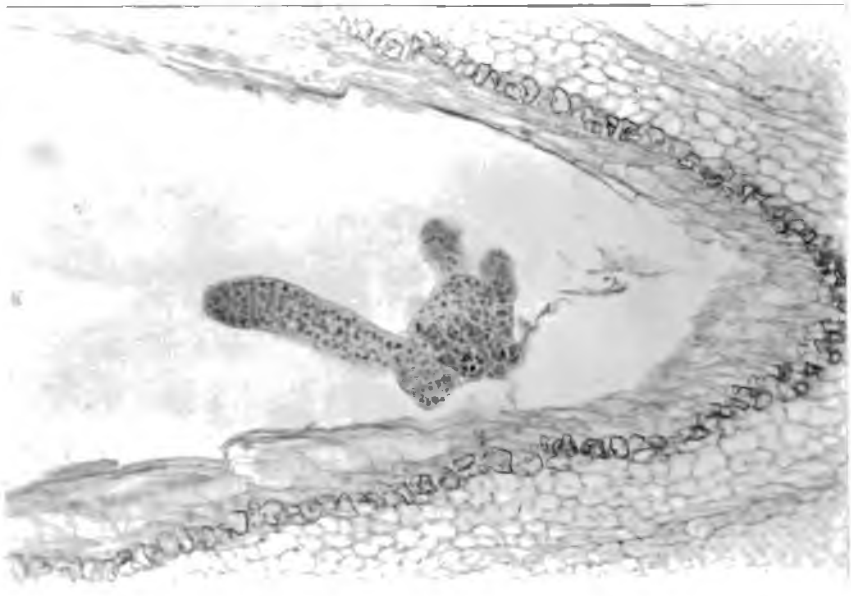

Figure 74

Carica cauliflora ovule 62 days after pollination by C. papaya, with budding cluster of embryos (X500)

lobes had elongated and appeared to have acquired a degree of polar differentiation.

On the 76th day, five of the twelve ovules sectioned were without embryos. The remaining seven ovules contained various polyembryonic structures similar to those seen at 62 days, but larger. Three of these embryo clusters showed signs of deterioration. Figure 75 shows a large polyembryonic cluster with many lobes, the largest of which appear to be slightly differentiated. No endosperm was detected. Pollen tubes were still present, but appeared to have collapsed in some cases, including two ovules with intact embryos. Figures 76-78 show three successive sections of one ovule containing a large undifferentiated embryonic tissue mass in close contact with an intact pollen tube. Figure 76 shows the pollen tube leading up to the embryo. Figure 77 shows a portion of the pollen tube extending past the end of the embryo. Figure 78 shows the pollen tube extending alongside the base of the embryo.

On the 90th day one of the nine ovules sectioned was empty. The remaining eight ovules contained either a mass of embryonic tissue or a cluster of embryos. Variability was more pronounced than at the earlier stages. Each of the ovules had a collapsed nucellus and lacked any endosperm. Pollen tubes were intact and in contact with the embryos in all but one of the ovules; the exception (Figures 79-81) was a collapsed ovule with degenerating embryo tissue and pollen tube. Figure 79 is a section of this ovule showing the collapsed walls and an elongated, abortive embryo. Close-up views of additional sections show the pollen tube leading from the micropyle (Figure 80) and the

Figure 75

Carica cauliflora ovule 76 days after pollination by C. papaya, containing cluster of connected embryos (X315)

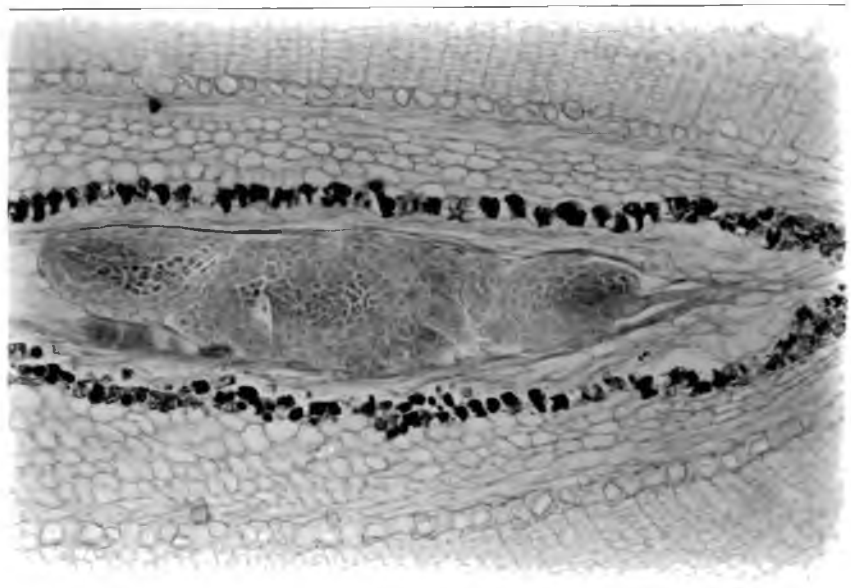

Figure 76

Carica cauliflora ovule 76 days after pollination by C. papaya, with undifferentiated embryonic tissue (X500)

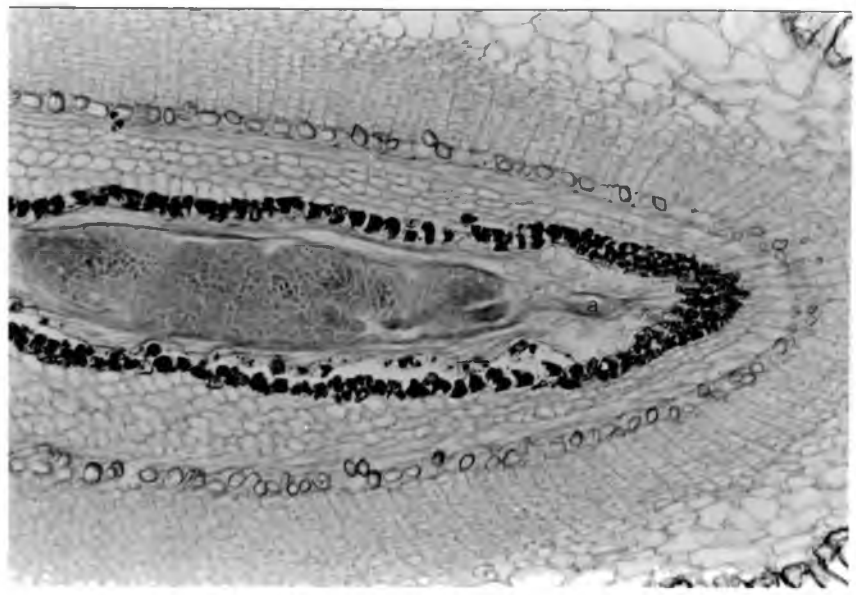

Figure 77

Carica cauliflora ovule 76 days after pollination by C. papaya, same ovule as in Figure 76; different section including pollen tube (a) (X315)

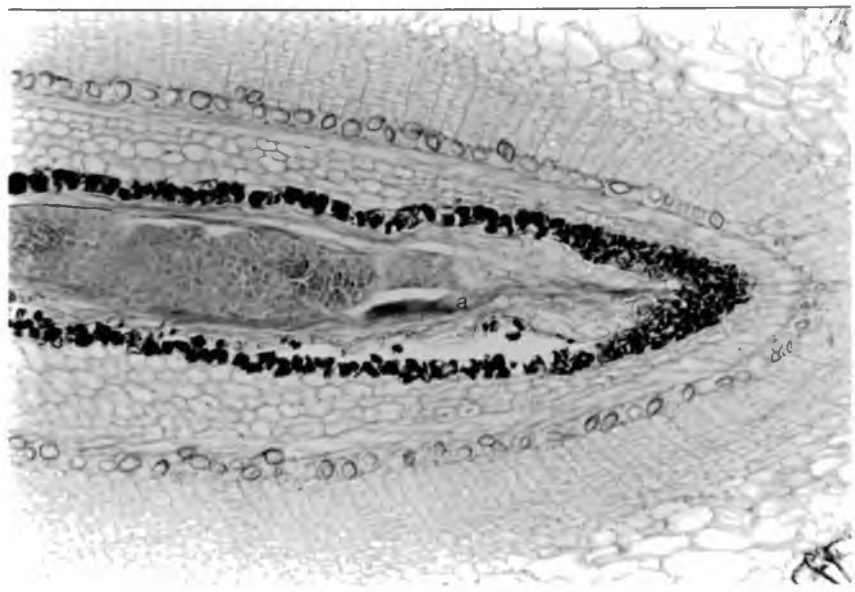

Figure 78

Same ovule as in Figure 77; adjacent section with pollen tube (a) extending alongside embryo (X315)

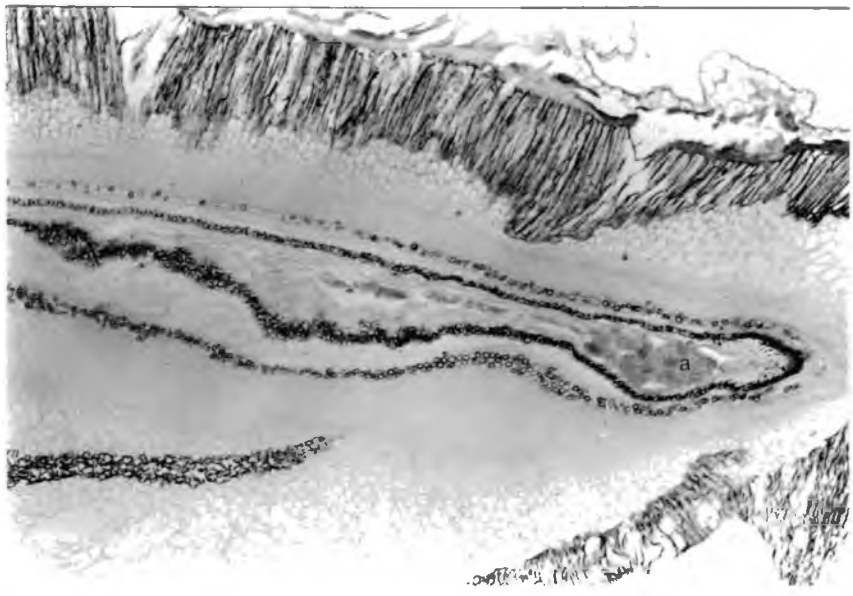

Figure 79

Carica cauliflora ovule 90 days after pollination by C. papaya, with collapsed interior and degenerating mass of embryonic tissue (a) (X125)

Figure 80

Carica cauliflora ovule 90 days after pollination by C. papaya, same ovule as in Figure 79; adjacent section with detail of collapsed pollen tube (a) (X500) (continued in Figure 81)

shrivelled end of the tube near the embryo (Figure 81). The seven remaining ovules contained large polyembryonic growths varying greatly in degree of differentiation. The least-differentiated embryo type is shown in Figure 82; although the embryonic tissue is heterogeneous with regard to cell morphology and fills well over half of the inner space of the seed, it is essentially a compact, callus-like mass lacking organized structures. This mass is in contact with the intact pollen tube shown at higher magnification in Figure 83. A polyembryonic growth exhibiting an intermediate level of differentiation is shown in Figure 84. Although the total embryo mass is somewhat smaller than in the previous case, there are many small, distinct, interconnected embryos showing some degree of polar differentiation. The highest level of embryo differentiation after 90 days, seen in two ovules, is shown in Figure 85. These embryo clusters did not have more total mass than the others, but some of the individual embryos had reached the heart-shaped stage with the cotyledon primordia visible. Suspensors could not be identified in any of the ovules.

Dissection of sixty-five fresh seeds from three mature fruits (153-172 days after pollination) produced forty seeds with well-developed integuments but no visible endosperm or embryo. The remaining twenty-five seeds also lacked endosperm, but did contain polyembryonic growths of various sizes and degrees of differentiation. The smallest were barely visible macroscopically, while the largest completely filled the area normally occupied by the embryo and endosperm. The least-differentiated polyembryos were much like those seen in the younger, sectioned material i.e., compact, callus-like masses of embryogenic tissue, except that some now filled the entire

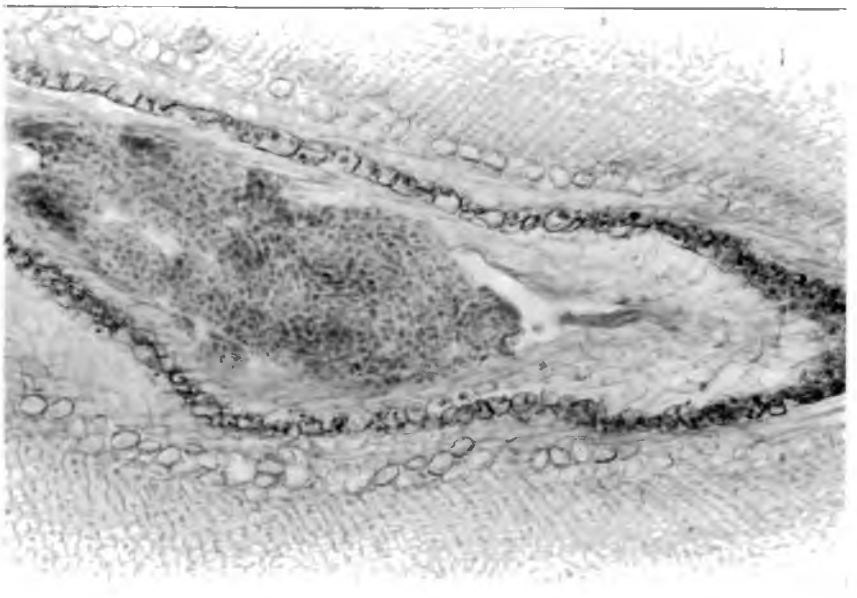

Figure 81

Carica cauliflora ovule 90 days after pollination by C. papaya, same ovule as in Figure 80; adjacent section including more of collapsed pollen tube (X500)

Figure 82

Carica cauliflora ovule 90 days after pollination by C. papaya, with undifferentiated embryonic tissue (a) (X315)

Figure 83

Carica cauliflora ovule 90 days after pollination by C. papaya, same section as in Figure 82; detail of intact pollen tube (a) in contact with embryo (X500)

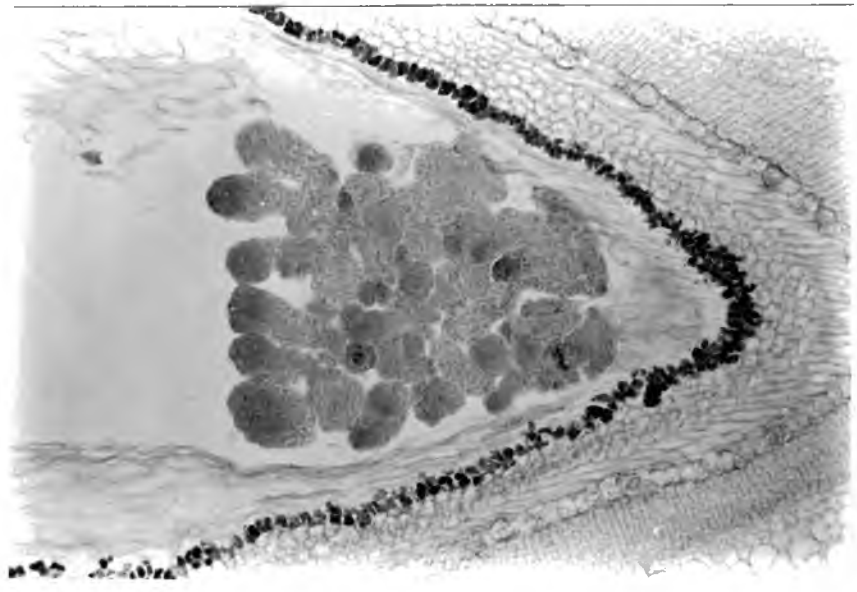

Figure 84

Carica cauliflora ovule 90 days after pollination by C. papaya, with polyembryonic cluster (X315)

Figure 85

Carica cauliflora ovule 90 days after pollination by C. papaya, with differentiated multiple embryos originating from single point (X315)

seed. The most-differentiated embryo clusters contained fully-developed embryos as large as those normally found in the parental species. Typically, one or a few of the highly-differentiated embryos in a cluster would be very large and the rest would be much smaller and less developed. Cotyledon asymmetry was common. The differentiated embryos were always oriented with the cotyledons at the chalazal end and the radicles converging at the micropylar end. In all cases where the embryonic tissue did not fill the seed, it was found concentrated at the micropylar end.

A comparative time chart of the results of the study of developing ovules is provided in Table 4.

TABLE 4. -- Comparative time chart of results of crosses

Cross	3 days	9 days	14-16 days
C. papaya X *C. papaya*	-intact synergids	-zygotes -no endosperm	-zygotes and 2-cell embryos -bulbous pollen tubes -free-nuclear endosperm
C. papaya X *C. cauliflora*	-intact synergids	-zygotes and intact eggs -little or no endosperm	-mostly intact eggs, some zygotes -few slender pollen tubes -no endosperm
C. cauliflora X *C. cauliflora*	-intact synergids	-intact eggs	-zygotes -slender pollen tubes -free-nuclear endosperm
C. cauliflora X *C. papaya*	-intact synergids	-pollen tubes in micropyles	-fertilization uncertain -bulbous pollen tube in nucellus

TABLE 4. -- (continued) Comparative time chart of results of crosses

Cross	22-23 Days	28-30 days
C. papaya X *C. papaya*	-embryos about 2 cells -increased free nuclear endosperm	-embryos 4-8 cells -increased free-nuclear endosperm -intact nucellus -intact pollen tube
C. papaya X *C. cauliflora*	-embryos 2 to several cells -no endosperm	-embryos 8+ cells -no endosperm -intact nucellus -intact pollen tube
C. cauliflora X *C. cauliflora*	-embryos about 2 cells -increased free-nuclear endosperm -intact nucellus	-embryos about 8 cells -free-nuclear endosperm -partly collapsed nucellus -intact pollen tube
C. cauliflora X *C. papaya*	-embryos about 4 cells -no endosperm -intact nucellus	-embryos 4-16 cells -no endosperm -enlarged nucellus -intact pollen tube

TABLE 4. -- (continued) Comparative time chart of results of crosses

Cross	44-45 Days	60-62 Days
C. papaya X *C. papaya*	-embryos about 16 cells -free-nuclear endosperm -intact pollen tube -nucellus intact but spongy	-embryos slightly larger -thickened free-nuclear endosperm -intact pollen tube -nucellus partly collapsed
C. papaya X *C. cauliflora*	-embryos irregular shapes <20 cells, some aborting -no endosperm -intact pollen tube -intact nucellus	-embryos variable, irreg., undiff., some aborted -scant or no endosperm -deteriorated pollen tube -spongy nucellus
C. cauliflora X *C. cauliflora*	-embryos club-shaped -free nuclear endosperm -intact pollen tube -collapsed nucellus	-embryos heart-shaped -cellular endosperm, 2/3 mature size -intact pollen tube
C. cauliflora X *C. papaya*	-embryos mostly aborted -no endosperm -intact pollen tube -enlarged nucellus	-polyembryony; variable size and differentiation -no endosperm -intact pollen tube -collapsed nucellus

TABLE 4. -- (continued) Comparative time chart of results of crosses

Cross	75-76 Days	90 Days
C. papaya X *C. papaya*	-embryos with polarity -endosperm becoming cellular -intact pollen tube -nucellus intact or not	-embryos with cotyledon primordia, variable size -early cellular endosperm -intact pollen tube -nucellus collapsed
C. papaya X *C. cauliflora*	-embryos variable, irreg., undiff.; some aborted -no endosperm -collapsed pollen tube -nucellus mostly intact	-embryos similar to 75 days -little or no endosperm -collapsed pollen tube -collapsed nucellus -collapsed inner integument
C. cauliflora X *C. cauliflora*	-cotyledons extending -endosperm mature size, but not dense -intact pollen tube	-embryos 3/4 mature size, fully differentiated -dense cellular endosperm -intact pollen tube
C. cauliflora X *C. papaya*	-polyembryony; >50% seeds empty or aborted -no endosperm -mostly intact pollen tubes	-polyembryony, variable size and differentiation, some with cotyledons -no endosperm -most pollen tubes intact

IV. DISCUSSION

COMPARISON OF POLLEN TUBE AND OVULE DEVELOPMENT AFTER INTRASPECIFIC POLLINATION OF *C. papaya* AND *C. cauliflora*

Pollen tube and ovule development in intraspecific-pollinated fruits of the two species were similar, the main difference being the rate of development. Although pollen tubes of the two species reached the ovules in the same amount of time, the interval from pollination to fertilization appeared to be a few days longer in *C. cauliflora* than in *C. papaya* flowers.

After fertilization, early development of ovules was similar until about the sixth week, when *C. cauliflora* began to show more rapid development.

The difference in rate of development between the two species increased through the 90th day after pollination. Endosperm in *C. cauliflora* enlarged and became cellular about two weeks earlier than endosperm in *C. papaya*. The difference in embryo development at 90 days after pollination was quite dramatic: *C. cauliflora* embryos had attained three-quarters of their mature size and were fully differentiated, while *C. papaya* embryos were barely approaching macroscopic proportions and did not have fully-formed cotyledons or radicles.

The difference in developmental rate between the species may have been influenced by seasonal differences, since *C. papaya* fruits at 62, 75 and 90 days after pollination were harvested in December, when nights were generally cooler and could have retarded growth, while the

same ages of C. cauliflora fruits were harvested in March and April when temperatures were generally higher. However, the 44-day-old C. papaya embryos were harvested in May, yet they were also smaller than the 45-day-old C. cauliflora embryos which were harvested in March when temperatures were somewhat cooler.

Another difference between the species was in the size of the pollen tubes. In C. papaya ovules, pollen tubes were usually very bulbous and contorted at the terminal end, especially the portion extending through the nucellus to the embryo. By comparison, C. cauliflora pollen tubes appeared narrower, less contorted and more delicate.

COMPARISON OF POLLEN TUBE AND OVULE DEVELOPMENT IN C. papaya X C. papaya AND C. papaya X C. cauliflora CROSSES

Pre-zygotic Development: Pollen tube behavior in C. papaya pistils was similar for pollen from C. papaya and C. cauliflora. The observation of pollen tubes of either species reaching the lowermost ovules of the ovaries in the same amount of time indicates that there is no pre-zygotic barrier involving inhibition of pollen tube elongation in C. papaya X C. cauliflora crosses.

Although pollen tube growth to the ovules was not inhibited, there was considerable variation in the time to fertilization in inter-specific pollinations, in contrast to more simultaneous fertilization in intraspecific pollinations. All ovules fixed 9 days after intra-specific pollination had zygotes, whereas interspecific pollinations

yielded a mixture of fertilized and unfertilized (i.e., intact synergids) ovules at 9 and 16 days after pollination. This suggests the possibility of an incompatibility mechanism which either retards or prevents discharge of the pollen tube contents and/or syngamy in some, but not all, ovules resulting from interspecific pollinations. Another relevant factor is the observation that in intraspecific pollinations of C. cauliflora, evidence of fertilization was not seen until 16 days after pollination; perhaps C. cauliflora pollen tubes simply require more time than C. papaya pollen tubes to complete fertilization. Ovules produced by interspecific crosses had all been fertilized 23 days after pollination. However, the ovules selected for sectioning were generally the larger ones, which would have been more likely to have been fertilized, so any partial inhibition of fertilization would not have been apparent due to the sampling technique used.

Post-zygotic Development: Post-zygotic development differed greatly in ovules produced by interspecific pollinations compared to those arising from intraspecific C. papaya crosses. Division of the zygote was apparent by 16 days after pollination in ovules resulting from intraspecific crosses, but not until the 23rd day in ovules containing hybrid embryos. However, intraspecific embryos and hybrid embryos had similar cell numbers at 30 and 45 days after pollination. Hybrid embryos showed abnormal growth with irregular shapes ranging from somewhat nodular to elongated or globose; signs of decline began to appear by 45 days after pollination. Some hybrid embryos remained alive and continued to grow slowly through 90 days after pollination,

and one had more than 100 cells after 75 days, but none differentiated significantly. The nodular shape of some hybrid embryos suggests an inclination toward polyembryony, as was seen to a marked degree in the reciprocal hybrid to be discussed in the next section. The proportion of declining and degenerating hybrid embryos increased over time. None of the older hybrid seeds dissected contained embryos large enough to be seen with the dissection microscope; apparently, intact hybrid embryos at 90 days after pollination were approaching the limit of their potential development. In contrast, C. papaya embryos had begun to differentiate after 75 days, and after 90 days most embryos had formed primary meristems and cotyledon primordia; development at these ages was not as advanced as it was in the material studied by Foster (1943) and Lamoureux (1955).

Endosperm development in C. papaya ovules fertilized in intraspecific crosses increased rapidly and became cellular in the third month as the embryos differentiated, in agreement with Foster (1943) and Lamoureux (1955). Most ovules developing after interspecific crosses lacked endosperm entirely, but three of them had highly abnormal, poorly developed, endosperm-like structures with dark-staining bodies that appeared to be enlarged nuclei. These observations suggest that while triple fusion may have occurred, followed by several mitotic divisions in at least some of the interspecific crosses, the endosperm nuclei apparently failed to undergo subsequent, normal mitoses and consequently, no normal endosperm formed.

The nucellus in ovules arising from interspecific crosses enlarged and remained fairly intact through the 75th day after pollination, even in ovules with aborting embryos. In C. papaya ovules fertilized in intraspecific crosses, the nucellus was declining by the 75th day as the endosperm was increasing. The nucellus had collapsed both in ovules containing intraspecific embryos and in those containing hybrid embryos by the 90th day. The earlier decline of the nucellus in ovules resulting from intraspecific pollinations may have been due to competition from the developing endosperm.

Pollen tubes remained intact in all of the C. papaya ovules produced by intraspecific pollinations through the 90th day after pollination. This differs from the observations of Foster (1943) and Lamoureux (1955) that pollen tubes collapsed at about the time that the endosperm began assuming a cellular structure, and before the rapid enlargement and differentiation of the embryo. The pollen tubes in ovules resulting from interspecific crosses appeared to begin declining as early as the 45th day, and by the 75th day only one intact pollen tube could be found; it was in contact with the largest embryo. The C. cauliflora pollen tubes observed in interspecific crosses were considerably less bulbous, straighter and more slender than those of C. papaya in intraspecific crosses; this is apparently characteristic of C. cauliflora (to be discussed further in the following sections).

The relationship between the collapse of the pollen tube and the degeneration of the embryo in C. papaya X C. cauliflora crosses is not clear from these results. While it is true that the largest embryo was accompanied by an intact pollen tube and the degenerating embryos were

associated with degenerating pollen tubes, it was not possible to determine whether the collapse of one structure caused the collapse of the other, or whether both collapsed because of a decline of the whole ovule due to more general factors, for example an incompatibility between the maternal tissue and the pollen tube and/or the embryo.

In summary, the results indicate the presence of a strong post-zygotic barrier to interspecific hybridization between C. papaya 'Washington' and C. cauliflora UH 345, using 'Washington' papaya as the female parent. The hybridization barrier is characterized by lack of endosperm development, premature pollen tube degeneration and abortion of hybrid embryos at a microscopic size prior to differentiation of organs.

COMPARISON OF POLLEN TUBE AND OVULE DEVELOPMENT IN C. cauliflora X C. cauliflora AND C. cauliflora X C. papaya

Pre-zygotic Development: Pollen tube behavior in C. cauliflora pistils was similar for pollen from both C. cauliflora and C. papaya. Pollen tubes of either species could be found extending to the lowest ovules in the ovaries 7 days after pollination. No pre-zygotic barrier involving inhibition of pollen tube growth to the micropyle was detected in C. cauliflora flowers pollinated by C. papaya. In fact, C. papaya pollen tubes were visible in the micropyles of C. cauliflora ovules after 9 days, while C. cauliflora pollen tubes were first seen in the 16-day specimens. This supports the suggestion in the previous

section that C. papaya pollen tubes may complete fertilization somewhat faster than C. cauliflora pollen tubes.

The presence of embryos in ovules resulting from interspecific crosses 22 days after pollination indicates that syngamy occurs, but the exact timing could not be determined from the material studied. The complete absence of endosperm in hybrid ovules at all ages studied could mean either that triple fusion does not occur or that interspecific triploid endosperm nuclei formed from triple fusion are unable to undergo mitosis.

Post-zygotic Development: As was the case in the reciprocal interspecific hybrid discussed in the previous section, post-zygotic development of ovules resulting from C. cauliflora X C. papaya interspecific crosses was very different than development of intraspecific-pollinated C. cauliflora ovules. At 22-23 days after pollination, both intraspecific and hybrid embryos had undergone only a very few divisions. At 28 days after pollination, variability became apparent among the hybrid embryos: some embryos were no larger than in the previous stage, while two embryos had at least sixteen cells each. In comparison, intraspecific embryos were about eight cells each at 30 days after pollination. At 44 days after pollination, only one of twelve ovules resulting from interspecific crosses contained an embryo; it was slightly smaller than, but similar to, the somewhat club-shaped, 45-day-old, intraspecific embryos. By 62 days after pollination, hybrid and intraspecific embryos had diverged sharply in development; the timing of the divergence suggests that abnormal development in

hybrid embryos is due, at least in part, to difficulty in undergoing normal differentiation. Hybrid embryos at 62, 76 and 90 days and at 6 months (ripe fruits) after pollination showed a strong tendency to become polyembryonic, regardless of whether differentiation occurred or not. The expression of polyembryonic character varied widely, including: large non-differentiated, somewhat nodular or lobed structures; clusters of many distinct, small globular bodies; and clusters of well-differentiated embryos. None of the differentiated hybrid embryos at 90 days after pollination were as advanced as intraspecific embryos of the same age, but at maturity some of the hybrid embryos were as large and as well-differentiated as intraspecific embryos. Although apparently sound hybrid embryos could be found at all ages, including mature fruit, it was clear that failure of hybrid embryo development was common, since ovules with no embryos, or with degenerating embryos, could be found at every developmental stage. This was true in spite of the fact that the seeds chosen for examination were generally the larger ones and would therefore have been the most likely to contain embryos. The largest sample of hybrid embryos was from ripe fruits, in which twenty-five of sixty-five seeds contained macroscopic embryos. The multiple embryos in each ovule appeared to be of zygotic origin, based on their location and orientation at the micropylar end of the ovule, as well as on the way they appear to have arisen from a single cluster of embryogenic cells.

Intraspecific C. cauliflora embryos showed no evidence of polyembryony. Intraspecific embryos at 62 days after pollination were more differentiated than the hybrid embryos, and by the 90th day after

pollination a rapid increase to two-thirds of mature size occurred, with complete differentiation of all embryonic organs.

Endosperm was totally lacking in all ovules derived from interspecific crosses. In contrast, free-nuclear endosperm was present 16 days after pollination in intraspecific-pollinated C. cauliflora ovules. This increased gradually until, after 62 days, a rapid increase in size and a conversion to cellular structure was underway. The rapid increase in endosperm in intraspecific-pollinated ovules occurred simultaneously with the rapid increase in embryo size. Lack of endosperm development may be a contributing factor in the failure of interspecific hybrid embryos, but other factors must also play a role, since at least some embryos can obtain sufficient nutrition from other sources to reach maturity. Perhaps hybrid embryos that succeed in developing derive their nutrition from the same source(s) as would normally nourish the endosperm. Whether the lack of endosperm in interspecific hybrid ovules is related to polyembryony cannot be determined from the results; although the two phenomena regularly occur together, there is no evidence of a cause and effect relationship. Although this study did not include a germination trial of mature hybrid seeds, several of the earlier works cited in the introduction reported that similar hybrid seeds were non-viable; lack of endosperm would explain the failure of germination, since the endosperm is clearly the primary nutrition source after the seed is separated from the parent plant.

Intact pollen tubes in contact with the embryo could be found in intraspecific-pollinated C. cauliflora ovules at all stages through

90 days after pollination. This finding supports Foster's (1943) suggestion for C. papaya that the pollen tube may play a role in providing nutrition for the developing embryo. However, pollen tubes remained intact after both embryo and endosperm were nearly mature, contrary to Foster's observation that the pollen tube collapsed as the endosperm and embryo began rapid development. This would indicate that the endosperm does not necessarily take over the nutritive role as the embryo undergoes rapid development. Further evidence for the possible nutritive role of the pollen tube comes from the C. cauliflora X C. papaya ovules: in general, surviving hybrid embryos, even very large ones at 90 days after pollination, were in contact with an intact pollen tube. The exceptions were two ovules at 76 days after pollination, with intact embryos but collapsed pollen tubes. If the pollen tube is required for nutrition of the embryo, the two apparent exceptions could represent cases where the pollen tubes recently collapsed and the embryos had not yet aborted. As discussed in the preceding section, the pollen tube behavior of the reciprocal hybrid, C. papaya X C. cauliflora, is difficult to interpret taken alone; however, it is consistent with the above suggestion for an important nutritive role for the pollen tubes, since surviving C. papaya X C. cauliflora embryos were associated with intact pollen tubes, while ovules without surviving embryos contained collapsed pollen tubes. There are evidently other factors in addition to pollen tube survival required for embryo survival, since C. cauliflora X C. papaya ovules often had intact pollen tubes without any apparent embryo, especially in ovules up to 62 days old.

The nucellus persisted until at least the 45th day after pollination in ovules derived from interspecific crosses, but only until the 30th day in intraspecific-pollinated ovules. The significance of this for the developing embryo is hard to assess, since most of the embryo development occurs after the nucellus has declined, regardless of pollen source. The cells in the nucellar beak, around the pollen tube, do persist longer and may help to nourish the embryo, either directly or via the pollen tube.

In summary, the results of C. cauliflora X C. papaya pollinations indicate the presence of a significant post-zygotic barrier to hybridization characterized by a complete lack of endosperm development, along with polyembryony apparently derived from the hybrid zygote, often accompanied by a total or partial lack of differentiation of embryos. The presence of well-differentiated, large embryos in some hybrid seeds suggests that it may be possible to obtain interspecific hybrid plants from some of the embryos by in vitro culture.

COMPARISON OF C. papaya X C. cauliflora WITH THE RECIPROCAL C. cauliflora X C. papaya

Interspecific hybrid embryos were obtained using either C. papaya or C. cauliflora as the female parent. However, when C. papaya was the female parent all of the embryos aborted at a microscopic size, without differentiation of organs. In contrast, when C. cauliflora was the female parent, the early development of the hybrid embryos was similar to that of the reciprocal hybrid, but some of the embryos of

C. cauliflora X C. papaya, rather than aborting, continued to develop and differentiate fully, despite undergoing polyembryonic multiplication. Other C. cauliflora X C. papaya embryos grew to a large size and remained sound in ripe fruits, but either failed to differentiate, or only partially differentiated.

The difference in degree of differentiation between the embryos of the reciprocal hybrids is probably due simply to the early abortion of the C. papaya X C. cauliflora embryos beginning around the 45th day; regardless of whether the embryos have the potential for differentiation, they abort before reaching the 75-day stage at which differentiation would first be expected.

There was a difference in the morphology of the pollen tubes in the hybrids. Pollen tubes of C. cauliflora in C. papaya ovules were smaller and appeared less robust than C. papaya pollen tubes in C. cauliflora ovules. If the pollen tube is a major source of nutrient supply for the developing embryo, then perhaps C. cauliflora pollen tubes in C. papaya ovules are not able to function adequately to sustain the embryos.

A possible cause of the failure of C. cauliflora pollen tubes in C. papaya ovules could be an incompatibility between the C. papaya ovular tissue and the C. cauliflora pollen tube, which causes premature collapse of the pollen tube.

Differences in survivability and maximum size attained, as well as degree of differentiation, between the reciprocal hybrid embryos could be due to cytoplasmic incompatibility, i.e., the hybrid nuclear genome may interact less favorably with cytoplasm derived from C. papaya egg

cells than with cytoplasm derived from C. cauliflora egg cells. Another possibility would be incompatibility between the hybrid embryo and surrounding maternal tissue; perhaps the hybrid is less compatible with C. papaya ovular tissue than with C. cauliflora ovular tissue.

There was no evidence of somatoplastic sterility or ovular tumors; these phenomena do not appear to play a role in the failure of either hybrid or in the difference between the reciprocals.

Normal endosperm was lacking in all hybrid ovules. Only a few C. papaya X C. cauliflora ovules showed any trace of endosperm, and that was very insubstantial. Therefore, differences in the genomic constitution of the endosperm do not appear to be responsible for the difference in success of the reciprocal hybrid crosses, even though the lack of endosperm may well be the single most important factor in the non-viability of interspecific hybrid seeds.

The variation in differentiation among C. cauliflora X C. papaya embryos at 62, 76, and 90 days post-pollination could have a genetic basis similar to the examples cited in the literature review (Hollingshead 1930, Sears 1944 and Stebbins 1958). According to this hypothesis, successfully differentiated embryos inherit an ideal combination of alleles to promote normal differentiation. Embryos of C. papaya X C. cauliflora could have the same genetic variation regarding the potential to differentiate, but their early abortion prevents expression of the trait.

V. PROPOSALS FOR FUTURE WORK

This investigation produced several important results which have possible application to future research directed toward production of Carica interspecific hybrids:

1.) Perhaps the most important finding is that large, differentiated embryos are formed by C. cauliflora X C. papaya crosses. This indicates the possibility of in vitro culture and germination of embryos to obtain interspecific hybrid plants. It is also noteworthy that the largest embryos were still sound in mature seeds; consequently, it would not appear to be necessary to rescue embryos for culture from immature ovules in order to escape premature abortion.

2.) The C. papaya X C. cauliflora combination used in this study failed to yield viable macroscopic embryos, yet earlier researchers (Jimenez and Horovitz, 1958; Khuspe et al., 1980; Litz and Conover, 1981) reported successful embryo formation using C. papaya as the female parent. This suggests that the early abortion of C. papaya X C. cauliflora embryos in this study may have been due to the specific combination of parental genotypes used. It would therefore be advisable to try a series of combinations of parental genotypes to evaluate combining ability for production of interspecific hybrids.

3.) Although C. papaya X C. cauliflora embryos aborted quite early, the fact that microscopic multi-cellular embryos formed occasionally suggests that in vitro ovule culture or embryo rescue techniques might allow the embryos to develop and subsequently

germinate into plants. (This would only be possible if the abortion of embryos *in vivo* were due to factors other than genetic disharmony in the embryo.)

4.) Lack of endosperm development is apparently a major factor in non-viability of interspecific hybrid seeds derived from *C. papaya* and *C. cauliflora*. Based on the hypothesis of endosperm balance number of Johnston et al. (1980) and on de Zerpa's (1958) observation of lack of endosperm in diploid X tetraploid intraspecific papaya crosses (see introduction), manipulations of ploidy levels in the parent species prior to crossing could be tried in an effort to produce interspecific hybrid seeds with sufficiently-normal endosperm and embryos to allow normal germination in soil, thereby eliminating the need for *in vitro* techniques. Various reciprocal interploid interspecific crossing combinations could be tested for endosperm development. Combinations of diploid and tetraploid would have the disadvantage of probable sterility in the triploid hybrid offspring. Combinations of diploid and hexaploid (if hexaploids could be obtained) would be expected to produce potentially-fertile tetraploid hybrids.

5.) The variability in degree of differentiation of the larger *C. cauliflora* X *C. papaya* embryos suggests a possible genetic basis for the degree of differentiation. Out of academic interest, one could test this hypothesis by developing highly inbred (i.e., homozygous) lines. If the degree of differentiation is genetically determined, then specific combinations of inbred parent lines would predictably produce embryos of the same degree of differentiation; conversely, if individual crossing combinations of inbred lines produced a wide range

of differentiation in the largest embryos, then the difference would more likely be due to non-genetic developmental factors.

Regarding formation of hybrid embryos and lack of endosperm development, the results of this study concur with the results of attempts at interspecific hybridization of C. papaya with C. cauliflora, C. microcarpa, C. monoica, C. pubescens (Jimenez and Horovitz, 1958) and C. stipulata (Horovitz and Jimenez, 1967). Therefore the preceding conclusions may be applicable to other sexually-incompatible crosses between C. papaya and wild Carica species.

VI. SUMMARY

There was no major pre-zygotic barrier to interspecific hybridization in either of the reciprocal hybrid crosses of C. papaya and C. cauliflora. The pollen tubes of each species penetrated the ovules of the other species without inhibition, and hybrid zygotes formed.

Substantial post-zygotic barriers to development of hybrids were observed in both reciprocals. The least-successful cross was C. papaya X C. cauliflora. Endosperm development was rare, and very atypical and under-developed when it did occur. Degeneration and abortion of embryos was evident after about 45 days and continued through 90 days, with no differentiation of tissues. The pollen tubes collapsed prematurely, compared to the pollen tubes in intraspecific crosses, which remained intact in the nucellus through 90 days after pollination. The reciprocal cross, C. cauliflora X C. papaya, completely lacked endosperm in all ovules, but pollen tubes generally remained intact during embryo development and some of the embryos attained a size and degree of differentiation similar to the mature parental species' embryos, without abortion.

There was a strong tendency for the C. cauliflora X C. papaya embryos to undergo polyembryonic multiplication, with the degree of differentiation ranging from virtually none up to fully differentiated. The hybrid embryos appeared to be of zygotic origin, based on several observations: the embryos were located in the

micropylar end of the embryo sac, where the egg apparatus had been; the serial sections showed a gradual sequence of development from zygote formation to embryogenic callus, which was followed by multiple embryo formation; finally, there was no indication of maternal tissue producing embryos.

LITERATURE CITED

Adsuar, J. 1971. Resistance of Carica candamarcensis to the mosaic viruses affecting papayas (Carica papaya) in Puerto Rico. J. of Agric. of Univ. of Puerto Rico. 55: 265-266.

Allard, R. W. 1966. Principles of Plant Breeding. New York: John Wiley and Sons, Inc.

Badillo, V. M. 1968. Monografia de la Familia Caricaceae. Facultad de Agronomia, Maracay.

Bannikova, V. P. and O. A. Khedynich. 1974. Features of fertilization in the course of remote hybridization of plants. In Fertilization in Higher Plants. Ed. H. F. Linskens. Amsterdam: North Holland Publishing Co.

Bennet, M. C., and F. W. Cope. 1959. Nuclear fusion and non-fusion in Theobroma cacao L. Nature. 183: 1540.

Brink, R. A., and Cooper, D. C. 1939. Somatoplastic sterility in Medicago sativa. Science. 90: 545-546.

Conover, R. A. 1964a. Distortion ringspot, a severe virus disease of papaya in Florida. Proc. Fl. State Hort. Soc. 77: 440-444.

Conover, R. A. 1976. A program for development of papayas tolerant to the distortion ringspot virus. Proc. Fl. State Hort. Soc. 89: 229-231.

Conover, R. A., and R. E. Litz. 1978. Progress in breeding papayas with tolerance to papaya ringspot virus. Proc. Fl. State Hort. Soc. 91: 182-184.

Cook, A. A., and F. W. Zettler. 1970. Susceptibility of papaya cultivars to papaya ringspot and papaya mosaic viruses. Plant Disease Reporter. 54: 893-895.

Cooper, D. C., and R. A. Brink. 1940. Somatoplastic sterility as a cause of seed failure after interspecific hybridization. Genetics. 25: 593-617.

Cooper, D. C., and R. A. Brink. 1945. Seed collapse following matings between diploid and tetraploid races of Lycopersicon pimpinellifolium. Genetics. 30: 376-401.

Currier, H. B. 1975. Callose substance in plant cells. Am. J. Bot. 44: 478-488.

Foster, L. T. 1943. Morphological and cytological studies on Carica papaya. Bot. Gaz. 105: 116-126.

Heslop-Harrison, J. 1975. Incompatibility and the pollen-stigma interaction. Ann. Rev. Plant Physiol. 26: 403-425.

Higgins, J. E., and V. S. Holt. 1914. The papaya in Hawaii. Hawaii Agric. Exp. Stn. Bull. 32.

Hollingshead, L. 1930. A lethal factor in Crepis effective only in an interspecific hybrid. Genetics. 15: 114-140.

Hogenboom, N. G. 1975. Incompatibility and incongruity: two different mechanisms for the non-functioning of intimate partner relationships. Proc. R. Soc. Lond. B188: 361-375.

Horovitz S., and H. Jimenez. 1967. Cruzamientos interespecificos e intergenericos en Caricaceas y sus implicaciones fitotecnicas. Agronomia Tropical. 17(4): 323-343.

Jensen, W. A. 1973. Fertilization in flowering plants. Bioscience. 23(1): 21-27.

Jimenez, H., and S. Horovitz. 1958. Cruzabilidad entre de especies de Carica. Agronomia Tropical. 7: 207-215.

Johnston, S. A., T. M. P. den Nijs, S. J. Peloquin and R. E. Hanneman, Jr. 1980. The significance of genic balance to endosperm development in interspecific crosses. Theor. Appl. Genet. 57: 5-9.

Kapil, R. N., and A. K. Bhatnagar. 1975. A fresh look at the process of double fertilization in angiosperms. Phytomorphology. 25: 334-368.

Khuspe, S. S., R. R. Hendre, A. F. Mascarenhas, V. Jagannathan., M. V. Thombre and A. B. Joshi. 1980. Utilization of tissue culture to isolate interspecific hybrids in Carica L. In National Symposium on Plant Tissue Culture, Genetic Manipulation and Somatic Hybridization of Plant Cells. Bhabha Atomic Research Center, Bombay, India.

Knox, R. B., A. Clark, S. Harrison, P. Smith and J. J. Marchalonis. 1976. Cell recognition in plants: Determinants of the stigma surface and their pollen interactions. Proc. Nat'l Acad. Sci. USA. 73: 2788-2792.

Kratzer, J. 1918. Die verwandtschaftlichen Beziehungen der Cucurbitaceen auf Grund ihrer Samenentwicklung. (Mit spezieller Berucksichtigung der Caricaceen, Passifloraceen, Aristolochiaceen, und Loasaceen.) Flora. 110: 275-343.

Laibach, F. 1925. Das Taubwerden von Bastardsamen und die kunstliche Aufzucht fruh absterbender Bastardembryonen. Z. Botan. 17: 417-479.

Lamoureux, C. H. 1955. Embryo development in papaya with reference to lethal factors. Unpublished M.S. Thesis. University of Hawaii.

Larter, L. Annual Report of the Dept. of Agriculture. Jamaica. 1937, p 76,77; 1938, p 88,89.

Lewis, D. and L. K. Crowe. 1958. Unilateral interspecific incompatibility in flowering plants. Heredity. 12: 233-256.

Litz R. E., and R. A. Conover. 1981. In vitro polyembryony in Carica papaya L. ovules. Z. Pflanzenphysiol. 104: 285-288.

Litz R. E., and R. A. Conover. 1982. In vitro somatic embryogenesis and plant regeneration from Carica papaya L. ovular callus. Plant Science Letters. 26: 153-158.

Maheshwari, P. 1950. An Introduction to the Embryology of Angiosperms. McGraw Hill, New York.

Malaguti, G., H. Jimenez y S. Horovitz. 1957. Pruebas de transmision del mosaico de la lechosa a otras especies de Carica. Agronomia Tropical. 7(1): 23-31.

Martin, F. W. 1959. Staining and observing pollen tubes in the style by means of fluorescence. Stain Technology. 34: 125-127.

McCray, F. A. 1933. Embryo development in Nicotiana hybrids. Genetics. 18: 95-110.

Mekako, H. and H. Nakasone. 1975. Interspecific hybridization among 6 Carica species. J. Amer. Soc. Hort. Sci. 100: 237-242.

Moore, G. A., and R. E. Litz. 1984. Biochemical markers for Carica papaya, C. cauliflora, and plants from somatic embryos of their hybrid. J. Amer. Soc. Hort. Sci. 109(2): 213-218.

Patil, L. K. and P. V. Wani. 1980. Varietal reaction of papaya against leaf blight caused by Helminthosporium rostratum Drechsler. Journal of Maharashtra Agricultural Universities. 5(3): 252.

Rappaport, J., S. Satina and A. F. Blakelee. 1950. Extracts of ovular tumors and their inhibition of embryo growth in Datura. Am. J. of Bot. 37: 586-595.

Reynolds, F. 1959. Papaya improvement in Honduras. Proc. Carib. Reg. Amer. Soc. Hort. Sci. VII Annual Meeting. p 57.

Ricelli, M. 1958. Resistencia al virus del mosaico y adaptabilidad de tres especies de Caricaceae. Agronomia Tropical. 13(2): 89-94.

Rietsema, J., S. Satina and A. F. Blakeslee. 1954. On the nature of the embryo inhibitor in ovular tumors of Datura. Genetics. 40: 424-431.

Sawant, A. 1958. Crossing relationships in the genus Carica. Evolution. 12: 263-266.

Sears, E. R. 1944. Inviability of intergeneric hybrids involving Triticum monococcum and T. aegilopoides. Genetics. 29: 113-127.

Shivanna, K. R. 1982. Pollen-Pistil Interaction and Control of Fertilization. In Experimental Embryology of Vascular Plants. Ed. B. M. Johri. Springer-Verlag.

Stalker, H. J. 1980. Utilization of wild species for crop improvement. Adv. in Agron. 33: 111-147.

Stebbins, G. L. 1958. The inviability, weakness and sterility of interspecific hybrids. Adv. Genet. 9: 147-215.

Storey, W. B. 1941. The botany and sex relationships in papaya. In Papaya production in the Hawaiian Islands. Hawaii Agric. Exp. Sta. Bull. 87: 5-22.

Storey, W. B. 1976. Papaya. In Evolution of Crop Plants. Ed. N. W. Simmonds. New York: Longman.

Subramanyam, M. D. and C. P. A. Iyer. 1982. Cytological studies in Carica spp. and hybrids. Genet. Agrar. 36(1/2): 73-86.

Traub, H. P. and C. J. O'Rork. 1939. Course of pollen tube growth in Carica papaya and Cucurbita sp. Nature. 143: 562.

Usteri, A. 1907. Studien uber Carica papaya L. Ber. Deutsch Bot. Ges. 25: 485-495.

Weaver, J. B. Jr. 1957. Embryological studies following interspecific crosses in Gossypium I. G. hirsutum X G. arboreum. Amer. J. Bot. 44: 209-214.

de Zerpa, D. M. 1958. Triploides de Carica papaya. Agronomia Tropical. 7: 83-86.